STUDIES ON THE BLOOD COPPER OF SHEEP AND OF COWS

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Published data for the copper content of the blood of normal sheep show values extending over a wide range.

TABLE 1.

Observer.	Range (mg. Cu per litre).
Tompsett (1934)	1·56–1·80
Bennetts and Chapman (1937)	0·64–0·87
Eden (1939)	0·16–1·64
Albiston *et al.* (1940)	0·8 –2·0
Innes and Shearer (1940)	0·47–2·81
Eden (1941)	0·13–2·10

Tompsett (1934) gives values of 1·80 to 2·23 mg. Cu per litre for ox blood, while Sjollema (1938) states that the normal figure for cattle is about 1·0 mg. per litre. Apart from the recent observations of Eden (1941) there seem to be no figures available concerning the effect of pregnancy on the blood copper level of sheep and cows, but it has been shown by Tompsett and Anderson (1935) that there is a rise in the copper content of the maternal blood of humans during the last few months of pregnancy.

The object of the present investigation was to obtain the range of blood copper values in normal sheep and cows under Western Australian conditions. The corresponding figures for haemoglobin were also obtained as existing data show a wide variation (see Underwood *et al.* (1939)). Collateral studies were also carried out on animals under conditions of naturally-occurring copper deficiency to provide information on the relation between low blood copper and haemoglobin levels.

EXPERIMENTAL.

Chemical methods. The blood copper of normal animals was estimated by the trichloracetic acid method of Tompsett (1934) using 20 ml. of blood and 50 ml. of the acid filtrate for analysis. Tests showed that no correction was necessary for the volume of the protein precipitate (cf. Thivolle and Laugier, 1938). Acid washed filter papers were used and the readings were made in an ordinary colorimeter with micro-cups.

With bloods of low copper content this method was used for routine analysis, a known amount of copper being added to bring the colour to a suitable intensity. In spite of obvious objections this procedure gives fairly accurate results (probably within ± 0·05 mg. Cu per litre). In the study of the relation between blood copper and haematopoiesis more precise values were necessary and the copper content was estimated by dry ashing 30–40 ml. of blood with magnesium nitrate (Van Niekerk, 1937) and extracting with dithizone as in the method of Sylvester and Lampitt (1935). With bloods of low copper content the accuracy of this method is probably ± 10 p.c.

Haemoglobin was estimated by the acid haematin method using a Newcomer disc which had been standardized against oxygen capacity (1 p.c. Hb = 1·34 vols. p.c. O_2).

Experimental animals. Observations were made on four groups of merino sheep grazing on natural pastures at Merredin in the drier portion of the Western Australian wheat belt: (1) 11 two-toothed wethers brought from Beverley just prior to the experiment, (2) 10 pregnant four-toothed ewes, (3) 4 ewes originally in group 2 which did not lamb, (4) 12 pregnant full-mouth ewes. The copper content of the natural pastures was found to fall from 10 p.p.m. Cu (dry basis) when young to 5–6 p.p.m. when mature. Green feed was only available to the above sheep on the last two samplings.

The results given in Table 3 were from mature Border Leicester-merino cross-bred ewes grazing at Beverley, Western Australia, where the pastures contain 7 to 13 p.p.m. Cu (dry basis)

during the growing period. This area appears to have a slightly higher copper status than Merredin. Green feed was available after the first sampling.

The Muresk cows (Table 5) were pure-bred Guernseys and the figures presented were a series of irregular determinations made on 17 cows over a period of 6 months (January to July, 1940). Samples were taken up to about two months before calving, and then from about one week after calving.

The Harvey cows (Table 5) were mainly Shorthorns and Jerseys. All samples were taken on 5th September, 1940. These animals were grazing on pastures which contained 9 to 14 p.p.m. (dry basis); these values are slightly higher than those usually encountered in Western Australia. The Perth animals were stall fed. Further details of the Muresk and Harvey cows have been described elsewhere (Beck, 1941).

The copper-deficient sheep (Table 6) were from Gingin (Bennetts and Beck, 1941) and the "Falling Disease" areas (Bennetts *et al.*, 1941). The deficient cows (Table 7) were all from the last areas. Pastures in these localities contain 1 to 3 p.p.m. Cu (dry basis). The figures given in Tables 7 and 8 are representative of a larger collection of data and have been selected as being typical of the blood changes which occur under conditions of naturally-occurring copper deficiency. It will be noted that in many cases there is a rise in blood copper in the late spring. This occurs generally in these areas, but the exact cause is unknown.

Results.

The data for normal cows and sheep are given in Tables 2, 3 and 5, and for deficient animals in Tables 6 and 7. Blood copper levels for sheep just before and after parturition are given in Table 4.

DISCUSSION.

The data for normal sheep in Western Australia confirm the findings of other workers that the blood copper levels for sheep under conditions of normal copper intake (5–10 mg. Cu per day) can vary within wide limits (0·4–1·6 mg. Cu per litre) without any effect on the health of the sheep. Most of the figures, however, lie between 0·6 and 1·2 mg. per litre. There is no apparent reason for the definite seasonal variation in the figures for the Merredin wethers, a variation which is quite absent in the ewes. The younger age of the wethers and the fact that these sheep previously came from an area of slightly higher copper status may be contributory factors, but no evidence is available to suggest that sex alone may produce such a difference.

The observation by Eden (1941) that the blood copper of individual sheep can vary very widely is amply confirmed by the results for the sheep of Table 2. The differences between the highest and lowest values for individual sheep were distributed as follows:

Range of differences. mg. Cu per litre.	Per cent. of wethers. Group 1.	Per cent. of all ewes. Groups 2, 3, 4.
0·22–0·39	18	46
0·40–0·59	73	35
0·60–0·78	9	19

There was, however, a definite tendency for sheep with higher or lower values to maintain their respective positions throughout the test.

Blood samples were taken from four of the sheep at 10 a.m. and 4 p.m. on three of the monthly tests. The values obtained in the morning were generally very slightly higher than those of the afternoon, but the difference was not significant.

There was no significant difference between the blood copper levels of the pregnant and non-pregnant ewes (Table 2). The values in Table 4 show that samples taken just after parturition show normal values. Samples taken one to four days before lambing generally show the same values as those obtained after lambing. Eden (1941) observed no rise in blood copper levels during the last few months of pregnancy.

The haemoglobin figures are generally slightly higher than those reported by Underwood *et al.* (1939) for sheep in another part of the Western Australian wheat belt.

TABLE 2.

Blood copper and haemoglobin levels of Merredin merino sheep.

(Blood Cu as mg. per litre; Hb as gm. per 100 ml.)

	3/11/39.	13/12/39.	17/1/40.	28/2/40.	27/3/40.	24/4/40.	22/5/40.	26/5/40.	31/7/40.	28/8/40.
Group 1. **Two-tooth wethers (eleven animals).**										
Blood Cu: Mean	1·03	0·95	0·78	0·88	0·73	0·75	0·66	0·66	0·76	0·88
Highest	1·23	1·06	0·96	1·03	0·87	1·16	0·88	0·90	1·03	1·34
Lowest	0·84	0·86	0·60	0·70	0·54	0·48	0·55	0·43	0·48	0·68
Haemoglobin: Mean	11·1	13·4	11·7	10·7	11·4	12·1	13·3	12·3	12·3	12·3
Group 2. **Four-tooth ewes. Pregnant (ten animals).**										
Blood Cu: Mean	0·96	1·05	0·93	1·14	0·86*	0·89*	0·86	1·00	0·97	1·09
Highest	1·09	1·15	1·03	1·52	0·99	1·11	0·98	1·33	1·21	1·29
Lowest	0·79	0·86	0·84	0·93	0·74	0·64	0·73	0·80	0·76	0·92
Haemoglobin: Mean	12·7	13·9	13·4	11·3	11·9	12·4	13·4	11·8	11·3	11·7
Group 3. **Four-tooth ewes. Non-pregnant (four animals).**										
Blood Cu: Mean	0·97	1·18	0·95	1·11	0·87	0·93	—	0·99	0·95	1·00
Highest	1·09	1·53	1·04	1·48	0·93	0·96	—	1·05	1·05	1·16
Lowest	0·84	1·02	0·89	0·94	0·79	0·87	—	0·93	0·82	0·77
Haemoglobin: Mean	13·4	14·9	13·7	12·3	12·9	13·7	—	14·1	13·2	12·8
Group 4. **Full-mouth ewes. Pregnant (twelve animals).**										
Blood Cu: Mean	—	0·99	0·94	1·02	0·82*	0·98*	0·82	0·92	0·94	1·11
Highest	—	1·40	1·27	1·61	1·17	1·18	1·00	1·10	1·16	1·44
Lowest	—	0·59	0·64	0·83	0·51	0·69	0·48	0·60	0·64	0·77
Haemoglobin: Mean	—	15·6	14·3	12·1	12·4	12·7	13·6	13·1	13·0	11·8

Group 1. Blood Cu. General mean = 0·81, S.D. ± 0·17. Range, 0·43–1·34. Difference for significance between monthly means = 0·11 (P = 0·01).
Haemoglobin. General mean = 12·1, S.D. ± 1·37. Range, 9·3–16·1.

Group 2. * Lambing between these two dates.
Blood Cu. General mean = 0·97, S.D. ± 0·15. Range = 0·64–1·52. Difference for significance between monthly means = 0·13 (P = 0·01).
Haemoglobin. General mean = 12·4, S.D. ± 1·27. Range = 10·1–15·6.

Group 3. Blood Cu. General mean = 0·99, S.D. ± 0·16. Range = 0·77–1·53. No significant difference between means.
Haemoglobin. General mean = 13·4, S.D. ± 1·20. Range = 11·5–17·0.

Group 4. * Lambing between these two dates.
Blood Cu. General mean = 0·95, S.D. ± 0·19. Range = 0·48–1·61. Difference for significance between means of months = 0·12 (P = 0·01).
Haemoglobin. General mean = 13·2, S.D. ± 1·81. Range = 9·9–17·4.

Blood copper of all Merredin sheep. Mean = 0·92, S.D. ± 0·19. Range = 0·43–1·61.
Haemoglobin of all Merredin sheep. Mean = 12·6, S.D. ± 1·57. Range = 9·3–17·4.

TABLE 3.

Blood copper and haemoglobin of seven cross-bred ewes.

(Blood Cu as mg. Cu per litre, Hb as gm. per 100 ml.)

Blood Cu: Mean	0·91	1·16	1·16	1·01	0·97
Range	0·98	1·45	1·46	1·14	1·17
	0·75	0·96	1·06	0·84	0·84
Haemoglobin: Mean	12·0	12·1	10·9	13·5	12·9

Samples taken 11/6/40 to 1/10/40 at four-weekly intervals. First sample taken two weeks after lambing.
Blood copper. General mean 1·04, S.D. ± 0·16. Range = 0·75–1·46. Difference for significance between monthly means = 0·18 (P = 0·01).
Haemoglobin. General mean = 12·3, S.D.± 1·28. Range = 10·0–14·2.

TABLE 4.

Blood copper of normal sheep near to parturition.

(mg. Cu per litre).

(Approximate hours of test before (—) or after (+) lambing given in brackets.)

Sheep No.	27/3/40.	10/4/40.	24/4/40.
B. 131	0·99	0·86 (+ 72)	0·80
B. 243	0·94	1·07 (+ 24)	0·89
V. 72	0·77	0·79 (+ 24)	0·92
V. 80	1·17	—	1·07 (+ 12)
B. 267	0·74 (— 24)	0·87	0·64
B. 244	0·74 (— 48)	0·94	0·95
V. 75	0·51	1·01 (— 96)	0·69
V. 77	0·81 (— 96)	0·92	1·18

TABLE 5.

Blood copper and haemoglobin levels of normal cows.

Locality		Muresk	Harvey	Perth
No. of observations		79	15	2
Blood Cu (mg. per litre):	Mean	0·97 ± 0·16	1·14 ± 0·27	0·98
	Range	0·69 — 1·47	0·82 — 1·66	0·97, 1·00
Haemoglobin (gm. per 100 ml.):	Mean	11·7 ± 1·11	14·9 ± 1·22	11·2
	Range	8·6 — 15·0	11·6 — 16·8	11·8, 10·6

TABLE 6.

Blood copper and haemoglobin levels of copper-deficient sheep.

Top figures, blood copper (mg. per litre): those by trichloracetic acid method in italics, others by dry ashing.
Lower figures, haemoglobin (gm. per 100 ml.).

Sheep No.	Period of test.	Breed and sex.†								
1	April–Nov., 1939	X E	*0·43*	*0·20*	0·19*	0·07*	0·07	0·10	0·12	0·17
			11·5	9·9	8·1	6·3	6·5	5·4	9·7	10·5
2	April–Nov., 1939	X E	*0·42*	*0·26*	*0·18*	*0·09**	*<0·05**	*0·14*	0·15	0·28
			12·5	12·1	11·3	12·7	11·1	10·5	10·9	12·5
3	April–Nov., 1939	X E	*0·50*	*0·12**	0·13*	0·14	0·06	*0·16*	0·15	0·23
			12·2	10·5	10·4	8·0	9·9	9·3	12·3	12·3
4	April–Nov., 1939	X E	*0·43*	*0·23**	0·19*	0·11	0·09	*0·17*	0·31	0·31
			13·2	9·6	11·2	10·6	8·5	9·9	11·5	12·6
5	March–Nov., 1939	M E	*0·27*	*0·34*	*0·18*	*0·10*	*0·07**	*<0·05**	0·09	*0·09*
			14·6	15·1	14·4	13·2	10·5	6·1	4·6	3·7
6	March, 1939–Aug., 1940	M E	*0·92*	*1·08*	*0·64*	*0·36*	*0·22*	*0·21**	*<0·05**	*0·14*
			13·2	11·7	13·7	13·2	13·4	7·9	9·1	7·6
			0·19	*0·41*	+	*0·33*	*0·27*	0·14*	0·14*	0·25
			9·0	9·8	+	12·2	12·6	11·0	9·4	6·5
7	May, 1939–Nov., 1940	M W	*0·31*	*0·18*	*0·05*	*0·22*	*0·11*	*0·16*	*0·16*	*0·14*
			14·2	13·9	13·2	13·6	12·7	10·5	12·7	14·1
			+	*0·22*	0·17	0·14	0·16	0·19	—	—
			+	13·7	11·4	13·4	11·9	8·3	8·4	11·0

Haemoglobin levels below 9·0 are regarded as evidence of anaemia, but only levels below 7·0 are considered to indicate severe anaemia.
Samples generally taken at monthly intervals.
* Sheep lambed between these two dates.
\+ Summer period (December to April) during which no samples were collected.
† E, ewe; W, wether; M, merino; X, Border Leicester-merino cross.

TABLE 7.

Blood copper and haemoglobin levels of copper-deficient cows.

Top figures, blood copper (mg. per litre): those by trichloracetic acid method in italics, others by dry ashing.
Lower figures, haemoglobin (gm. per 100 ml.).

Cow No.	Period of test.								
1	May, 1939–	*0·35*	*0·23*	*0·15*	*0·16*	*0·12**	0·17*	0·11	*0·08*
	Sept., 1940	10·2	11·8	11·0	12·6	12·0	9·0	8·9	11·9
		+	*0·33*	*0·42*	0·36	0·36	*0·25*	0·18	—
		+	11·4	10·8	10·2	10·7	11·0	5·2	6·5
2	May, 1939–	*0·37*	*0·21*	*0·19*	*0·19*	*0·29*	0·12	0·14	*0·05*
	Sept., 1940	9·8	11·2	12·2	12·8	11·5	10·1	11·0	13·6
		+	*0·11**	0·44	0·36	0·17	0·29	0·12	—
		+	10·6	10·2	10·0	9·9	8·6	5·4	7·1
3	May, 1939–	*0·20*	*0·21**	*0·50**	*0·39*	*0·29*	0·18	0·22	*0·15*
	Sept., 1940	11·0	11·9	10·8	11·1	10·0	9·5	10·5	11·7
		+	—	*0·42*	*0·30*	*0·41*	*0·26*	0·16	—
		+	10·4	10·2	9·9	9·8	10·5	4·7	8·3
4	May, 1939–	*0·25*	*0·38*	*0·20*	*0·17*	*0·09*	0·17	0·09	*<0·05**
	Sept., 1940	10·4	11·8	12·7	12·6	11·3	9·2	10·8	12·8
		+	—*	*0·37*	*0·16*	0·24	0·20	0·09	—
		+	—	9·2	8·9	9·9	8·8	4·7	7·2
5	May–December,	*0·17*	*0·15*	*0·15*	*0·11*	*0·07*	0·11	0·16*	0·09*
	1939	9·0	9·2	9·4	11·0	9·5	7·7	9·7	10·3

Haemoglobin levels below 9·0 are regarded as evidence of anaemia, but only levels below 7·0 are considered to indicate severe anaemia.
Samples generally taken at monthly intervals.
Cow 1 received supplement of Fe, Co, Ni, Zn and Mn (but no Cu) during first eight tests.
* Calving between these two dates; Cow 2 calved on day of sampling.
\+ Summer period (December to March) during which samples were not collected.

The blood copper levels of cows also range within fairly wide limits (0·69 to 1·66 mg. per litre). No seasonal variation was noted in these figures.

Schultze *et al.* (1936) have made studies on the blood of copper-deficient pigs and reached the conclusion that a minimum blood copper level of 0·20 mg. per litre was necessary for haemoglobin formation. Their experiments are hardly comparable with those given in the present paper, but our findings in copper-deficient sheep and cows are as follows: The blood copper level can remain at 0·2 to 0·3 mg. per litre for long periods without any occurrence of anaemia. There is no absolute correlation between low blood copper levels and the development of anaemia, but if the blood copper level falls below 0·1 to 0·2 mg. per litre for any length of time anaemia generally develops. Further discussion is unwarranted in view of our limited knowledge of the function of copper in haemoglobin formation.

SUMMARY.

The copper content of the blood of normal Western Australian sheep varies between 0·4 and 1·6 mg. Cu per litre; most figures however lie between 0·6 and 1·2 mg. per litre. Haemoglobin figures for the same sheep lie between 9·3 and 17·4 gm. per 100 ml.

Normal cows show blood copper values between 0·7 and 1·7 mg. Cu per litre, and haemoglobin figures between 8·6 and 16·8 gm. per 100 ml.

In copper-deficient sheep and cows there appears to be no absolute correlation between low blood copper levels and the development of anaemia, but anaemia

generally occurs if the level falls below 0·1 to 0·2 mg. Cu per litre for any length of time.

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REFERENCES.

Albiston, H. E., Bull, L. B., Dick, A. T. and Keast, J. C. (1940): Austral. Vet. J., 16, p. 233.
Beck, A. B. (1941): Austral. J. exp. Biol., 19, p 145.
Bennetts, H. W. and Beck, A. B. (1941): Coun. Sci. and Ind. Res. (Aust.) Bulletin (in press).
Bennetts, H. W. and Chapman, F. E. (1937): Austral. Vet. J., 13, p. 138.
Bennetts, H. W., Beck, A. B., Harley, R. and Evans, S. T. (1941): *Ibid.*, 17, p. 85.
Eden, A. (1939): J. comp. Path. and Therap., 52, p. 249.
Eden, A. (1941): J. Agric. Sci., 31, p. 186.
Innes, J. R. M. and Shearer, G. D. (1940): J. comp. Path. and Therap., 53, p. 1.
Schultze, M. O., Elvehjem, C. A. and Hart, E. B. (1936): J. biol. Chem., 116, p. 107.
Sjollema, B. (1938): Biochem. Z., 295, p. 372.
Sylvester, N. D. and Lampitt, L. H. (1935): Analyst, 60, p. 376.
Thivolle, L. and Laugier, P. (1938): C.R. Soc. Biol., 128, p. 1210.
Tompsett, S. L. (1934): Biochem. J., 28, p. 1544.
Tompsett, S. L. and Anderson, D. F. (1935): Brit. J. exp. Path., 16, p. 67.
Underwood, E. J., Harvey, R. J. and Beck, A. B. (1939): Austral. J. exp. Biol., 17, p. 193.
Van Niekerk, P. LeR. (1937): Onderstepoort J. Vet. Sci., 9, p. 623.

THE OCCURRENCE OF LIPOID STRUCTURES IN CULTURES OF *STREPTOBACILLUS MONILIFORMIS*[1]

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Strangeways (1933) demonstrated by dark-ground illumination that broth cultures of *Streptobacillus moniliformis* contained many striking and unusual structures in addition to the ordinary bacterial elements. These structures consisted of spherical globules in foam-like masses, varying in size and luminosity and floating amongst the bacterial threads. Klieneberger (1936) also referred to this peculiarity and regarded it as an entirely characteristic feature of the *Streptobacillus moniliformis*, distinguishing it from all other known types of bacteria.

No special study has been made of these structures. Klieneberger considered them as essentially belonging to the L.1 organism which in turn she regarded as a symbiont occurring in *Streptobacillus moniliformis*, and suggested that they might represent a secretion of this organism. During an investigation of several strains of streptobacillus recovered in a naturally occurring epizootic among wild mice (Williams, 1941), this property was examined in detail.

METHODS.

The two strains upon which these observations were made were obtained from naturally infected wild mice. One corresponded closely to the published descriptions of the organism, while the other showed certain morphological differences, having a more persistent mycelial stage and a greater tendency to form swellings and nodes on solid media. Their pathogenicity for white mice was lower than is usually recorded, producing arthritic lesions with death in three to five weeks. Both possessed an equal and striking capacity to produce the structures noted by Strangeways.

Since *Streptobacillus moniliformis* requires for its growth the incorporation of serum in the media, cultures were made using nutrient broth and agar to which 30 p.c. to 50 p.c. of sterile normal horse serum had been added immediately before inoculation. Such media gave a good and characteristic growth, enabling the strains to be maintained for more than seventy subcultures. In these cultures colonies which resembled Klieneberger's descriptions of the L.1 organism were occasionally observed, but all attempts to isolate them in a pure state failed. The technique of Klieneberger was followed, and although repeated efforts were made with both strains, subcultures invariably reverted to the standard streptobacillary type. In view of the recent work of Dienes (1939), in which the L.1 organism was shown to be a variant rather than a symbiont, it was decided, for the purposes of this work, to regard cultures as consisting essentially of one specific organism.

At first, microscopic examinations were made with dark-ground illumination but it was soon found more convenient and satisfactory to use a wet film preparation examined by transmitted light with high power or oil immersion lenses.

The Appearance of the Structures in Cultures.

When the flocculent deposit from a forty-eight hour broth culture was observed with a high power lens many structures were found which never appeared in stained films and which differed markedly from the twisted bacterial mycelium. They were seen as spherical, ovoid, cylindrical or finger-like bodies, quite transparent with a sharply defined edge, varying in size from about 3 to 30μ. They existed singly, in small clumps or in large foam-like masses, floating freely in the medium or enmeshed in the bacterial threads. Some bodies were denser than others, and showed a refractile double-contoured edge. Occasionally small granules were seen adhering to the outside surface. Their fluid consistency could readily be discerned from the way bodies altered their shape when they came into contact with bacterial masses or with one another. They were heavier than water, being deposited with centrifuging, and remained stable in strong acid and alkaline

[1] Since this work was completed, a paper by Partridge and Klieneberger, recording similar findings, has appeared in the Journal of Pathology and Bacteriology of March, 1941.

solutions, in distilled water, hypertonic saline and on heating to boiling. They were, however, destroyed, as Klieneberger noted, by the application of some of the common fat solvents. If a film were allowed to dry under the microscope, the bodies were seen to disintegrate and disappear completely. Subsequent staining of the film revealed only scattered granular debris where the bodies had been. In fluid preparations they would take up some bacterial stains such as Giemsa and methylene blue, to a very limited extent, providing only a faint contrast with the background.

In addition to these globular forms, many thin filaments were found which were clearly distinct from the bacterial threads. These appeared to be associated with the globules, and frequently arose from a cluster and ran perfectly straight across several fields to end in other bodies. This uniform straightness was in marked contrast to the irregular twisting of the bacterial threads. More rarely, a filament would contain a well-defined obtuse angle at one or more points along its length. In diameter they varied from short cylindrical processes of 10μ across to long drawn-out threads barely visible with the oil immersion lens. Some filaments showed small oval expansions without breaking their continuity, while in others sudden alterations of diameter occurred. An interesting process was observed when the coverslip was gently pressed down on the slide. With the increasing pressure many of the globular bodies became squeezed out and took up the form of filaments. As the pressure relaxed some of these filaments remained drawn out while others steadily withdrew to form again the globular bodies. It was thus seen that the spherical globules and the thin straight filaments were fundamentally the same structures.

Substantially the same appearances were obtained with cultures grown on solid media. Colonies picked off and emulsified with saline were found to contain identical globular and filamentous bodies. Direct observations on agar plates revealed the presence of globules all through the colony and they were especially noticeable at the periphery. The slight pressure produced by placing a coverslip on top of a colony was often sufficient to transform these globules into filaments.

The Nature of the Structures.

While in some respects the bodies resembled protoplasmic elements there were a number of features which did not agree with this view. It was found that they disintegrated on drying and could not be demonstrated in stained films. Yet if a dried smear were emulsified with water, they reappeared with all their specific forms. Klieneberger's observation regarding the destructive powers of alcohol, ether and chloroform was confirmed, the addition of these solvents to a culture resulting in the instantaneous disappearance of all globules and filaments therefrom. If dried films were treated with these solvents, subsequent emulsification with water failed to produce the bodies. It was further found that if several loopfuls of culture were shaken in a small quantity of alcohol and the alcohol then added to water, a fine white precipitation occurred, indicating that some water-insoluble substance had been extracted.

In order to examine this extracted substance a broth culture was spun in an angle centrifuge at 2,000 r.p.m. for fifteen minutes, and the deposit washed twice with saline to remove traces of the medium constituents. This deposit was dried in a dessicator and thoroughly extracted with successive quantities of acetone and alcohol. Evaporation of the acetone fraction yielded abundant cholesterol crystals, while from the alcohol fraction a soft yellowish fatty substance was obtained, which was shown to possess the physical and chemical properties of lecithin. Together, these two fractions appeared to form about half the total bulk of the dried culture. This procedure was repeated several times upon cultures of both streptobacillus strains, and gave the same results in every case.

When a small portion of the lecithin-containing substance was examined microscopically it was found to be a greasy amorphous material bearing no resemblance to the structures seen in cultures. With the addition of a drop of water, however, a surprising change took place. This was the well-established phenomenon of the production of the myelin forms of lecithin, in which the amorphous material extruded from its edges thin cylindrical processes showing peculiar slow writhing movements. By gentle pressure and maceration these twisting interlaced fingers could be transformed into the globules and extremely fine filaments so characteristic of the *Streptobacillus moniliformis* cultures. Similar manipulations with the myelin forms of lecithin prepared in the usual way from egg yolk and horse serum yielded structures whose specific appearance and behaviour were identical in every way with the culture bodies. A sample containing equal parts of cholesterol and lecithin, which had been intimately mixed by solution in the same solvent and subsequent evaporation to dryness, was observed to behave with regard to the production of myelin forms, in the same fashion as lecithin alone. There was no visible evidence of the presence of cholesterol although it could readily be recovered by the selective solvent action of acetone. The same effect resulted when the total residues of the acetone and alcohol fractions of cultures were thoroughly mixed and added to water, showing that, in the presence of water, the whole of the extracted lipoid fraction was capable of existing in the form of myelin bodies.

Many careful comparative examinations were made of the culture structures and artificial lecithin preparations, and the clear demonstration of such unique properties in common could leave little doubt that the appearance in each case arose from the same phenomenon.

The Origin of the Lipoid Material.

It was of some importance to determine whether the lipoid material was a synthetic product or whether it resulted from the action of bacterial metabolism upon the serum lipoids present in the medium. As the organism would not grow in the absence of serum, it was necessary to procure a serum from which the lipoids had been artificially removed. Following the technique of Hewitt (1927) a sample of horse serum was prepared which retained all normal constituents except those soluble in ether or alcohol. In practice, this method has been found to yield an entirely fat- and lipoid-free product. When broth and agar media, enriched with this serum, were inoculated with streptobacillus strains a ready growth was obtained, microscopic examinations of which showed no evidence of lipoid structures; the bacterial filaments appeared normal in every way, and were the only visible elements in the cultures. The total amount of growth obtained with this serum was somewhat less that that usually found with whole serum, but in view of the complete absence of lipoid bodies such a reduction was to be expected. Subcultures of the lipoid-free organisms into whole serum broth resulted in the reappearance of the lipoid bodies as usually seen. Further corroborative evidence of this finding was obtained from similar experiments with serum globulin and small quantities of whole serum. In the process of therapeutic serum concentration carried out in these Laboratories, approximately 95 p.c. of the total phospholipid and cholesterol is eliminated from the final pseudo-globulin fraction. Suitable proportions of this substance added to ordinary media gave a reduced but adequate growth of the streptobacillus with a complete absence of the lipoid bodies. It was also found that a reduced growth without the formation of bodies occurred when only small amounts (1–3 p.c.) of whole serum were employed. In both these cases the bacterial chains were the only visible elements.

From these results it was seen that the process was directly associated with the presence of lipoids in the medium, and, as a good growth was obtained with fat-free serum, it could be concluded that the production of lipoid structures was a non-essential part of the metabolism of the organism. For this reason detailed chemical investigations of the culture lipoids were not included in this work. A superficial examination showed the presence of cholesterol and a lecithin complex in the rough proportions of 1: 3. Occasional fat globules were found, and it is highly probable that other serum lipoid substances were also included.

A small number of quantitative estimations was carried out to show the general trend of the process. Broth cultures containing varying amounts of serum were inoculated with the same strain of streptobacillus and incubated for 48 hours. The washed deposit from each culture was transferred to a weighed watch glass, dried, and repeatedly extracted with acetone and alcohol. The final weights of the residue and extracted fractions were then ascertained. These figures are given in Table 1. It was found that approximately half the dry weight of culture deposits was composed of lipoid materials. This weight and also that of the bacterial residue were dependent upon the amount of serum present, although the proportionate values of each were maintained in cultures varying considerably in their serum content. The deposited lipoid did not represent the total quantity in the available serum but was about one-quarter of this figure, suggesting that an equilibrium was reached where the reaction ceased.

TABLE 1.

The proportion of lipoid material in cultures.

Volume of culture.	Per cent. of serum in cultures.	Weight of dry culture deposit.	Weight of lipoid fraction.	Per cent. of lipoid in dry culture deposit.
c.c.		mg.	mg.	
80	33	49·9	25·9	51·9
100	50	81·2	36·0	44·3
150	50	104·0	50·9	48·9
200	50	173·5	91·8	53·2
275	33	165·6	80·7	48·7
475	40	341·1	176·7	51·8

The factor responsible for this phenomenon was not present in bacteria-free filtrates of cultures. When Seitz "EK" filtrates from cultures containing either whole serum or fat-free serum, were incubated with fresh serum broth, no change occurred. Similar negative results were obtained when suspensions of the bacteria were added to fresh serum broth and incubated under conditions which did not permit growth. These suspensions were obtained from fat-free serum cultures as it was necessary, in order to detect the specific lipoid changes, to use organisms initially free from lipoid bodies. With cultures grown in whole serum the bacteria could only be freed from bodies by the repeated application of fat solvents, a procedure likely to destroy any active principle. Suspensions were added to whole serum broth, and incubated at 37° C. for 48 hours, growth being prevented firstly by the addition of 1 p.c. of toluene or 0·5 p.c. of phenol, and secondly by using organisms which had been allowed to die out naturally. No lipoid changes

occurred under these conditions, and only during bacterial growth was it possible to demonstrate the formation of the lipoid bodies. These experiments have not been carried far enough to determine the exact mechanism by which the lipoids are freed from the serum but the evidence suggests a bacterial endo-enzyme which has the power to disrupt some protein-lipoid linkage.

DISCUSSION.

It is hoped that the findings presented will lead to a better understanding of this property of the *Streptobacillus moniliformis*. So far as is known it is without an exact parallel in bacteriology, although the studies of Klieneberger (1938) suggest that a similar phenomenon may occur with certain pleuropneumonia-like organisms. Examination of photographs taken by Turner (1935) of the development of the organism of pleuropneumonia itself, particularly those designated as the Genethode IV phase, also leads to the conclusion that lipoid structures were in part responsible for the appearances. Since all these organisms depend for their growth on the addition of serum to the usual laboratory media, it may be a function common to them as a group. That the property is not a general accompaniment of all bacterial growth in serum media may be assumed from the fact that Strangeways and Klieneberger both regarded it as quite specific for the streptobacillus. This point was further borne out when an unsuccessful search was made in the course of this work amongst pyogenic, intestinal, soil and air-borne bacteria for the development of lipoid bodies in the presence of serum.

The physico-chemical changes underlying the process are necessarily speculative. The method by which lipoids are carried in serum is little understood and Marrack (1938) summarizes the position by offering three possible mechanisms: an adsorption of the lipoid on protein, a surrounding of the lipoid with a protein film, or a chemical union of the two. Whichever system actually operates, there must be a constant need for adjustments depending upon the physiological requirements of the body. Such adjustments are presumably brought about by complex chemical actions within the body, whereby lipoid substances are combined with, and detached from, the circulating serum proteins. The fact that this bacterium effects just such a change in the normal course of growth may provide opportunities for the closer study of this aspect of lipoid metabolism.

There are few references in the literature to investigations of the behaviour of lecithin in water. Leathes (1925) described a series of experiments with photographs showing the effects of various ions on the formation of the myelin bodies. His hypothesis was that a combination of localized imbibition and surface growth, i.e., a "negative surface tension", took place in the production of the bodies. These properties resulted from the particular affinity for water of certain component groups of the lecithin molecule. This peculiarity affords a useful means of recognizing lecithin or mixtures containing it when they occur in the presence of water.

The exact significance of Klieneberger's detailed studies on the L.1 organism is not quite clear. Although she regarded it as a symbiont, she admitted that her findings did not exclude the possibility that it was a variant form of the *Streptobacillus moniliformis*. Later work by Dienes (1939) confirmed her observations as to the existence of a separate colony type, but presented evidence to show that it was essentially a variant and would, under suitable conditions, revert to the standard streptobacillary form. The descriptions and photographs published by both these workers, while containing certain minor discrepancies, make it plain that a fundamental feature of the L.1 organism is the production of lipoid structures. Indeed, if these structures are discounted it is difficult to see what actual protoplasmic elements are present. Klieneberger's diagrams and photographs of stained preparations showed fine filamentous networks which Dienes, using a different technique, was not able to demonstrate. It is possible that

the presence of relatively large quantities of lipoid material may have brought about misleading appearances. It was found, for example, that dried films of prepared lecithin, when stained with Giemsa or methylene blue, showed areas of fine myceliał networks which closely resembled these photographs.

Further work will doubtless clear up these points and serve to define more accurately the relationship of the L.1 organism and of the *Streptobacillus moniliformis* to the production of lipoid bodies, and to each other.

SUMMARY.

The structures associated with the bacterial elements in cultures of *Streptobacillus moniliformis* are found to be lipoid in nature, and represent the myelin forms of lecithin.

They are derived from the serum incorporated in the culture media.

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REFERENCES.

Dienes, L. (1939): J. infect. Dis., 65, p. 24.
Hewitt, L. F. (1927): Biochem. J., 21, p. 216.
Klieneberger, E. (1936): J. Path. Bact., 42, p. 587.
Klieneberger, E. (1938): J. Hyg., 38, p. 458.
Leathes, J. B. (1925): Lancet, 208, p. 957.
Marrack, J. R. (1938): Med. Res. Council, Sp. Rep. Series No. 230.
Strangeways, W. I. (1933): J. Path. Bact., 37, p. 45.
Turner, A. W. (1935): *Ibid.*, 41, p. 1.
Williams, S. (1941): Med. J. Austral., 1, p. 357.

TECHNIQUE OF ADRENALECTOMY IN THE GUINEA-PIG AND THE MAINTENANCE OF THE ADRENALECTOMIZED ANIMALS

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The development of the technique of adrenalectomy in the guinea-pig recently became necessary in this Department to enable a study to be made of the behaviour of these animals when completely adrenalectomized and subsequently injected with doses of thyreotrophic hormone.

Though regularly practised in other species, adrenalectomy in the guinea-pig has received scant attention. Simmons and Whitehead (1936) were the first to describe a technique enabling complete surgical removal of the adrenals in guinea-pigs. Schachter and Bebee (1939) later described a modified technique, whilst the present investigation was being conducted.

In this communication a technique adapted from that of Simmons and Whitehead is described, which has been found suitable for the preparation of moderately large animal groups. The procedure is detailed for each side, since each presents its own difficulties.

The problem of maintenance of the adrenalectomized animals is also considered from the point of view of providing adequate hormone replacement, and dietary and mineral requirements.

EXPERIMENTAL PROCEDURE.

Material. Immature guinea-pigs of both sexes belonging to either one of two stocks were used at varying ages for the operation. During the later stages of the experiments, sexes were segregated (when animals weighed more than 300 gm.). The animals were maintained always on a liberal diet of bran, fresh lucerne and water, in a room artificially heated during the winter months to a temperature of 20–24° C. Routine necropsies were performed on all adrenalectomized animals; any tissue suspected of being of adrenal nature was submitted to histological examination.

General factors. Complete adrenalectomy was performed in two stages at an interval of a week or more, and the right side, being more difficult, was operated on first. Animals were fed right up to the time of operation. Open ether anaesthesia was employed.

Technique of adrenalectomy on right side (animal's head away from operator).

1. A 3 cm. skin incision was made over the last rib from a point just short of the midline posteriorly.

2. The parietes were cut immediately above and below the rib, which was then divided at the outer border of the spinal muscles. The abdomen was opened, exposing the liver, intestines and right kidney.

3. The liver was retracted upwards, the kidney posteriorly, and the intestines packed off anteriorly. This exposed the field of operation.

4. The connective tissue between the suprarenal gland and the vena cava was put on the stretch and divided with a fine sharp hook from above downwards, thus freeing the gland from the lateral border of the vena cava. Gauze dissection increased the separation.

5. The connective tissue binding the suprarenal vein to the vena cava, at the superomedial angle of the gland, was pierced from the lateral to the medial side, the hole gently enlarged and the suprarenal vein clamped for one minute before cutting it. Bleeding was slight and easily staunched. The separation of the remainder of the superior border of the suprarenal from the liver and diaphragm was completed with scissors. The posterior surface of the suprarenal was separated so far as possible at this stage from the tissues of the posterior abdominal wall.

6. The anterior surface of the suprarenal was next separated from the kidney by blunt dissection.

7. The anteromedial aspect of the gland was freed from the vena cava by careful gauze dissection from above downwards. Tougher strands of connective tissue were severed with a sharp

[1] The above investigation was performed during the tenure of the Marion Clare Reddall Scholarship, as part of a research programme generously aided by a grant from the National Health and Medical Research Council.

hook. It was essential at this stage to keep tissue planes on the stretch, to allow definition of planes of cleavage.

8. The gland, remaining attached only on its posteromedial aspect to the tissues of the posterior abdominal wall, was then easily removed. The suprarenal artery, which became divided in this operation, did not require ligation.

9. The gland was checked for completeness after removal, and a search made for accessory adrenal tissue. The latter when present occurred along the course of the vena cava and usually near the pelvis of the kidney. It was removable without difficulty.

10. The parietes were closed in one layer with continuous 000 catgut. Interrupted skin sutures were used, and a collodion dressing applied to the wound.

11. Risks during the operation were:

(i) Opening the right pleural cavity in the angle between the eleventh rib and the lumbar muscles.
(ii) Injury to the vena cava.
(iii) Tearing the surface of the suprarenal gland. The latter was therefore on no account touched or lifted with forceps.

Technique of adrenalectomy on left side (animal's head toward operator).

1. A 3 cm. skin incision was made parallel to but just below the last rib, extending from a point just short of the midline posteriorly.

2. The parietes were incised just below the last rib, which was not removed. The spleen, intestines, and left kidney were exposed in the wound.

3. The spleen and intestines were packed off. Retraction of the kidney posteriorly then exposed the suprarenal gland.

4. Separation of the gland was begun at its inferior border by dividing the connective tissue between it and the anterior aspect of the left renal vein. The suprarenal vein entering the medial border of the gland near its lower end was avoided at this stage.

5 The lateral border of the gland was stripped off the anterior surface of the kidney from below upwards, and the posterior surface of the gland freed as far as possible.

6. Separation of the upper pole of the gland and the upper part of its medial border from the posterior abdominal wall was accomplished.

7. With tissues on the stretch, the gland was freed in its lower posterior portion, in the angle between the pelvis of the kidney, posterior abdominal wall, and left renal vein. This divided the suprarenal artery, which did not require ligation.

8. The inferior surface of the gland was separated from the left renal vein and adjacent tissues of the posterior abdominal wall by careful gauze dissection. The gland remained attached only by the suprarenal vein, which was clamped for one minute before dividing.

9. The gland was checked for completeness after removal, and a search made for accessory adrenal tissue.

10. The operation was completed as on the right side.

11. The risks involved during the operation were similar to those on the right side, but not so great.

Maintenance of Completely Adrenalectomized Animals.

In these investigations the stock diet of bran and fresh lucerne employed in this Department proved quite satisfactory for the adrenalectomized guinea-pigs, as one would expect from the predomination of carbohydrate in its composition (cf. Halnan and Garner, 1940). The animals uniformly showed a steady daily weight increase, though this was not so large as for normal animals. Preference for lucerne was usually shown, and dry lucerne was used as a convenient adjuvant to the moist. Cod-liver oil 0·2 c.c. was administered twice weekly.

As regards salt therapy, Bomskov (1939) advocated a daily intake of 0·3 mg./gm. for small animals. It was decided in these experiments to administer daily per os, 1 c.c. of a solution containing 10 p.c. sodium chloride and 0·8 p.c. sodium citrate, to ensure that each animal received its requisite dosage of sodium and chloride with a slight excess of the former over the latter.

Specific Hormone Replacement Therapy.

The dosage of the various adrenal cortical extracts and preparations available to maintain life in adrenalectomized animals has not been determined for guinea-pigs. Small animals generally, however, require more extract per unit weight than larger, since the requirements appear to have a definite relation to the surface area (Bomskov, 1939), doubtless because of the influence of the latter on heat loss. Schaechter and Bebee (1939) reported that the average equivalent required daily by 1 kg. of adrenalectomized guinea-pig was 45 gm. of fresh whole adrenal tissue, and that the requirements for individual guinea-pigs were fairly constant. For rats Bomskov stated that the daily equivalent of adrenal tissue required varied from 30–120 gm. Assuming a guinea-pig to weigh 600 gm., the requirements for this animal would appear to be approximately one-half to one-third those of the rat.

A limited supply of the very active synthetic compound desoxycorticosterone acetate (DOCA) recently prepared by Steiger and Reichstein (1937) was available. This has been shown to be ten

times as potent as the most active crystalline fraction from cortical extracts (corticosterone), in maintaining the life of adrenalectomized rats (Simpson, 1939). No experimental data were available concerning the minimum effective maintenance dose for guinea-pigs, and the supply of DOCA available would not permit the performance of a controlled assay experiment. However, from experience accumulated during the progress of the experiments, it was apparent that a single daily injection of 0·2 mg. DOCA given subcutaneously, the daily dosage eventually adopted, was adequate to maintain adrenalectomized guinea-pigs in a state of health for a period of at least three weeks. Actually this dosage was probably in excess of the minimal requirements, since adrenalectomized animals receiving additional large doses of thyreotrophic hormone daily were maintained in good health for a period of two weeks, after which period they were deliberately sacrificed (Robinson and Trikojus).[2]

The DOCA used ("Cortenil")[3] was supplied in ampoules of 1 c.c. containing 5 mg. DOCA dissolved in sesame oil. The contents of each ampoule were diluted 10 times with peanut oil before injection and the prepared solution kept constantly in the refrigerator. All injections were warmed by hand before use, and given to the animals subcutaneously on the ventral aspect. An area of induration developed at the site of each injection and lasted for some 10–12 days, so that a fresh site was chosen for each day's injection. The first injection for each animal was given on the morning following the second operation.

RESULTS.

Occurrence of accessory adrenal tissue. In all, 96 guinea-pigs were completely adrenalectomized. Seven of these possessed accessory adrenal bodies, six of which were on the right side along the course of the vena cava (usually at its lower part) and one on the left side in connection with the left renal vein. In addition, one accessory adrenal body was found on the right side at necropsy, apparently arising by hypertrophy from a smaller amount of accessory tissue missed at operation. This represented an incidence of accessory tissue in 8·5 p.c. of cases.

Data concerning operation. The mortality at operation was severe at first, but as experience was gained in the technique, fell progressively. In the final group of 29 guinea-pigs, 2 succumbed at operation, representing a mortality of 7 p.c. The principal cause of death was anaesthesia, next in importance being injury to the vena cava. Fully-grown animals were not used because it was desired to study the effects of thyreotrophic hormone injections subsequently, but the rule applied that the older the animal, the easier was the operation to perform. The time taken for the operation was 20–25 minutes for the right side, and rather less on the left side.

Post-operative recovery was very rapid, the animals showing a tendency to feed within 3–4 hours of operation. Normal activity was restored by the following morning. Post-operative deaths were uncommon but occasionally resulted from shock or post-operative pneumonia.

Post-operative survival and maintenance. The post-operative course in animals not receiving daily injection with DOCA was not studied. However, in a group of 9 adrenalectomized animals used as controls for another experiment and which were injected for 4 days only with the customary dosage of DOCA, 7 died within 8 days of the cessation of injections, but the remaining two survived for two weeks, when they were deliberately sacrificed. Those dying exhibited the usual signs of acute adrenal insufficiency before death. Examination for adrenal tissue revealed a microscopic piece in one of the animals which survived for eight days only, but in none of the others.

The post-operative course when the animals were maintained by injections of DOCA and adjuvant dietary measures has been summarized for the large groups studied, in Table 1.

In the animal groups studied by both Simmons and Whitehead, and Schachter and Bebee, all adrenalectomized animals died within seven days of the second operation, so that the table quoted provides adequate figures to demonstrate the efficacy of DOCA in maintaining the life of adrenalectomized guinea-pigs. The dosage of DOCA adopted in the last three experimental groups (0·2 mg. per day) was adequate for the adrenalectomized animals within the weight groups studied (220–370 gm. at time of second operation), since only eight out of thirty-nine animals

TABLE 1.

Showing survival in experimental groups of adrenalectomized guinea-pigs maintained with DOCA.

Sex.	Total No.	Average weight at time of second operation (gm.).	Animals dying within 8 days of complete adrenalectomy.
M	28	326	1
F	37	317	5
Total	65	321	6

Note. A total of 21 of these animals (9 M, 12 F) received in addition thyreotrophic hormone injections commencing between the 4th and the 8th days following the second operation.

[2] To be published.

[3] Grateful acknowledgment is made here to Bayer Pharma Ltd., Sydney, who donated, in part, the Cortenil used in these experiments.

failed to survive for at least two weeks after operation; three of these eight were receiving in addition daily doses of thyreotrophic hormone. Younger animals were found to succumb more readily, despite the administration of full doses of DOCA. In our experience guinea-pigs weighing 250 gm. or more at the time of the second operation were most suitable.

Postmortem findings. At necropsy, following either natural death or sacrifice, the wounds were regularly found to have healed completely. Adhesions over the site of operation were frequent. A careful search for residual adrenal tissue was made in every adrenalectomized animal. Tissue suspected of being of adrenal nature was found in 47 of the 96 cases, and removed for histological examination. One of these tissue-pieces was the accessory adrenal body already mentioned as found postmortem; three others of the remaining 46 specimens did show residual adrenal cortical tissue, in a state of degeneration; only one of the three positive specimens occurred in the last two experimental groups comprising 40 guinea-pigs. The only valid means of excluding the presence of any adrenal tissue is to take serial sections of the connective tissue from the entire adrenal area, but this was deemed unnecessary in the present series of experiments when complete removal of the adrenal glands had already been verified at operation.

DISCUSSION.

The results extend the observations of Simmons and Whitehead, and Schachter and Bebee, on the effects of adrenalectomy in the guinea-pig. The technique of adrenalectomy here adopted resembles that of the former investigators rather than of the latter. The operative shock produced in Schachter and Bebee's series was probably less, as claimed by the authors, than in Simmons and Whitehead's series, since the average survival period (4·5 days) was definitely better in the case of the former investigators; but it is felt that the manipulations to which they objected of drawing the kidney into the wound, and dividing the rib on the right side, are not accompanied by undue shock if the operation is expeditiously performed, and that the original technique is less likely to leave any glandular remnants behind.

The finding of a definite incidence of accessory adrenal bodies in guinea-pigs is in contradistinction to the results of Schachter and Bebee, who found no accessory tissue in their series of 50 guinea-pigs. The results obtained in this work indicate that the guinea-pig is no exception to other mammalian species studied in regard to the presence of accessory adrenal tissue, although its incidence may perhaps be less. Accessory tissue, too, would appear always to be situated near the main glands. It may be mentioned that these findings are supported by observations made in this Department during routine postmortem of several hundred guinea-pigs, in which an incidence of accessory adrenal tissue in approximately 5 p.c. of cases has been noticed.

SUMMARY.

A technique for completely adrenalectomizing guinea-pigs in two stages is described.

The maintenance of adrenalectomised guinea-pigs in a state of apparent health by dietetic and specific hormonal treatment is discussed.

The efficacy of desoxycorticosterone acetate in maintaining life in adrenalectomized guinea-pigs is demonstrated, and a satisfactory daily dosage (probably not the minimal) of this preparation established.

Acknowledgments. I desire to express my thanks to Mr. W. S. Stephen and Mr. W. J. Ellis for technical assistance during the performance of the operations. I am grateful to Professor C. G. Lambie and Dr. V. M. Trikojus for their encouragement and criticism.

REFERENCES.

Bomskov, C. (1939): "Methodik der Hormonforschung", Thiele, Leipzig, 1, pp. 495–6.
Halnan, E. T. and Garner, F. H. (1940): "The Principles and Practice of Feeding Farm Animals" (figures quoted from supplementary tables reproducing those in Bulletin of Ministry of Agriculture).
Schachter, R. J. and Bebee, M. O. (1939): Proc. Soc. exp. Biol. Med., 40, p. 541.
Simmons, H. T. and Whitehead, R. (1936): J. Physiol., 88, p. 235.
Simpson, L. (1939): Proc. Roy. Soc. Med., 32, p. 685.
Steiger, M. and Reichstein, T. (1937): Nature, 139, p. 925.

STUDIES IN THE METABOLISM OF PLANT CELLS

1. ACCUMULATION OF CHLORIDES BY PLANT CELLS AND ITS RELATION TO RESPIRATION

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INTRODUCTION.

During the last 20 years a large amount of data has been accumulated on the absorption of inorganic ions by plant cells. The problem, however, is still without a satisfactory explanation though the electrochemical theory which has recently been put forward by Lundegårdh (1940b) offers a plausible interpretation of many of the phenomena observed. The mechanisms suggested by Lundegårdh may be valid for tissues other than those with which his data were collected. Even if this prove to be the case the problem still remains of connecting the chemical changes which liberate energy to bring about the accumulation of salts with the chemical changes known to occur in normal respiration. Lundegårdh's suggested mechanism is not acceptable to Hoagland and Steward (1939, 1940), who doubt the reality of the anion respiration coefficient on which Lundegårdh's conception largely depends. Lundegårdh's theory has been further criticized by Steward and Preston (1941), who found that salt accumulation in potato discs occurs only if protein synthesis takes place.

In the course of investigations into the effect of the accumulation of various chlorides upon respiration in carrot tissue, the present writer has obtained data which bear directly upon the general theory of salt respiration. Some of these data were transmitted to Nature (Robertson, 1940) following the controversy between Lundegårdh (1939, 1940a) and Hoagland and Steward (1939, 1940). The interest of these results is that they corroborate Lundegårdh's general findings in a quite distinct type of tissue.

The importance of the relationship between the accumulation of various salts and tissue respiration has been recognized since Steward (1932) showed that the amount accumulated was dependent upon the supply of oxygen to the tissue. Since then Steward and Hoagland have repeatedly emphasized that there is a connection between aerobic respiration and the rate of accumulation. Further, Lundgårdh and Burström (1933), Steward (1937), and Steward, Stout and Preston (1940) have shown that there is an effect of salts upon the carbon dioxide production of the tissue. Lundgårdh and Burström and Steward found a difference between the reaction to chlorides in the two types of tissue they used. Lundgårdh and Burström (1933) found that all chlorides increased carbon dioxide output of wheat roots while Steward (1937) and Steward and Preston (1941) found that respiration in potato tissue was decreased during the accumulation of calcium chloride. It is reasonable to suppose that different tissues will respond in different ways to the same salt. It was decided to investigate the effects of various chlorides upon the respiration of carrot tissue with a view to obtaining more information about the relationship between salt accumulation and respiration. The writer has already used carrot tissue for salt accumulation experiments at the Botany School, Cambridge (Briggs and Robertson, unpublished).

In order to avoid confusion in terminology it is desirable to define the words "absorption" and "accumulation". "Absorption" is used by the writer to mean the entry of salts into a tissue by any mechanism. "Absorption" may comprise "exchange", which is the entrance of an ion from the external solution to replace an ion lost by the tissue, and "accumulation" which is the entrance of both ions of a salt by a process not yet explained. "Salt respiration" or "extra carbon dioxide production" is the term given to that increment of respiration which accompanies the entrance of a salt from the external solution.

MATERIAL AND METHODS.

The tissue used in these experiments was xylem parenchyma from carrot roots cut into discs 1·5 cm. in diameter and 0·1 cm. in thickness. After cutting the discs were threaded on a non-corroding wire, each disc being separated from its neighbour by a glass bead. The discs were then washed in running tap water (or in some cases in aerated distilled water which was changed every few hours). After washing for various periods the discs were rinsed in distilled water and put into the absorption vessels.

The absorption vessel consisted of a long tower in which 80 discs, threaded on wire, could be placed in 240 ml. of liquid. Constant conditions of aeration were maintained by a continuous stream of carbon dioxide free air in fine bubbles from a scintered disc in the bottom of the tower; since the discs were separated by glass beads their surfaces maintained contact with the aerated liquid. The gas stream passed from the absorption tower through a tube attached to the top by a ground glass joint. This tube was removed when the discs were being inserted. The absorption of ions was followed by measuring changes in the conductivity of the external solution; a conductivity cell into which a sample of the solution could be withdrawn was in contact with the liquid in the absorption tower.

After passing over the tissue and leaving the absorption tower the gas stream passed into specially constructed vessels containing 0·1 M NaOH in which the carbon dioxide, evolved by the tissue, was absorbed. Efficient absorption was secured by passing the gas into the NaOH through a scintered bubbler and then circulating gas and alkali together. The amount of carbon dioxide evolved by the tissue during a given period was determined from the change in conductivity due to the formation of Na_2CO_3.

The whole apparatus for salt absorption and carbon dioxide estimation was immersed in a thermostat which consisted of a large volume of water controlled by a gas thermo-regulator. Constant temperature was maintained within $\pm$ 0·05° C. Particular care was taken to determine the exact temperature and to correct for any small variations during readings of conductivity.

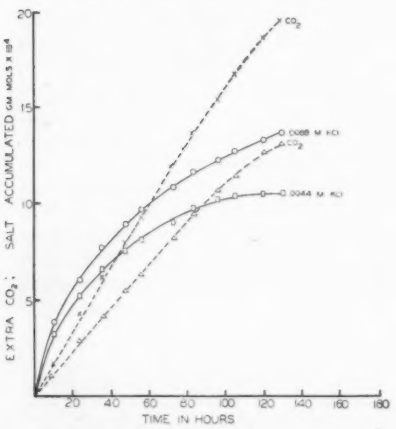

Fig. 1. Amounts of potassium chloride accumulated and of extra carbon dioxide evolved, plotted against time, for tissue placed initially in solutions of 0·0088 M KCl and 0·0044 M KCl.

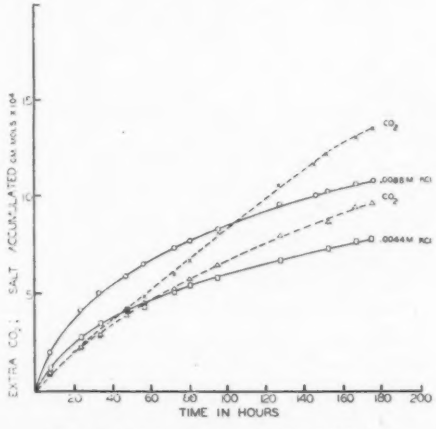

Fig. 2. Amounts of potassium chloride accumulated and of extra carbon dioxide evolved, plotted against time, for tissue placed initially in solutions of 0·0088 M KCl and 0·0044 M KCl.

It is well known that the transfer of discs of storage tissue from the washing water to distilled water generally results in an increase in the conductivity of the external liquid over the first few hours, followed by a decrease of conductivity to values which, in some cases, are almost as low as that of conductivity water. In the series of experiments to be described the sets of tissue were always put into distilled water initially, and the conductivity of the external solution was allowed to fall to a steady value before the electrolyte was added. In this way the rate of salt accumulation in the tissue, as measured by the disappearance of ions from the external solution, was not interfered with by the initial loss of electrolyte from the tissue.

The advantages of the conductivity technique of measuring ion absorption are that it provides a rapid method of estimating ionic concentration and that it does not interfere either with the composition or volume of the external solution. The limitations of the technique are realized; particularly that it gives an estimate of the ionic concentration but no indication of the ionic com-

position. Since the object of these experiments is to investigate the effect of accumulating ions on the respiratory activity this is considered no disadvantage since it automatically eliminates estimating those ions which enter the tissue by exchange for ions coming out into the external solution. Numerous workers have shown that there is a difference in the amounts of anion and cation which are absorbed but it is established that this difference can be accounted for by the exchange of ions already present in the tissue.

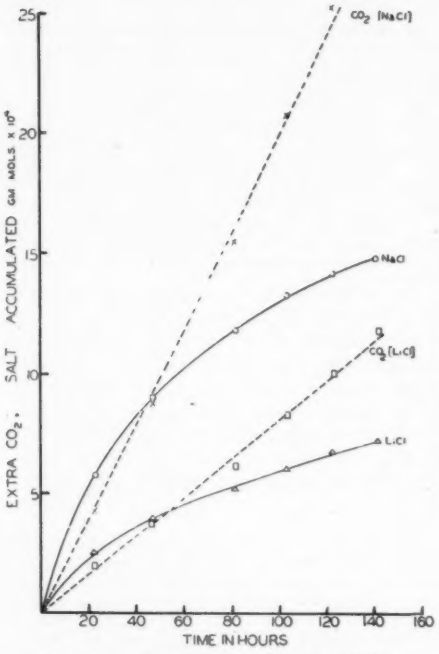

Fig. 3. Amounts of sodium and lithium chlorides accumulated and of extra carbon dioxide evolved, plotted against time, for tissue placed initially in solutions of 00108 M NaCl and 0·0072 M LiCl respectively.

RESULTS.

The Accumulation of Potassium Chloride.

Two series of experiments were made, each with two different concentrations of potassium chloride. In the first of these experiments, which was done at a temperature of 23·5° C., three sets of tissue were used. After the tissue had equilibrated with the distilled water, during which time the respiration fell to a steady value, potassium chloride in a concentration of 0·0088 M[1] was added to one set of tissue and a concentration of 0·0044 M to another set of tissue. The third set of tissue was left in distilled water as a control. Assuming that the decrease in conductivity is due to the accumulation of potassium chloride from the external solution, it is possible to estimate the amount of potassium chloride accumulated. In both sets of tissue to which the potassium chloride was applied there was a marked increase in the rate of carbon dioxide production above the level maintained by the control. The amount of potassium chloride accumulated and the carbon dioxide evolved in excess of that produced by the control are shown, plotted against time, in Fig. 1.

From these results it is clear (a) that accumulation was most rapid immediately after the addition of the salt, and that the rate of accumulation subsequently fell and approached a steady rate in the case of the more concentrated solution; the rate fell similarly in the dilute solution and approached zero when practically all the ions had been removed from the external solution, and (b) that the respiration showed an immediate increase to a new level after the application of the salt and maintained that level with very little reduction in rate as long as the salt was being accumulated; there was a slight reduction in rate in the more dilute solution after accumulation had almost ceased.

This experiment was repeated with tissue from another source using the same salt (potassium chloride) at the same temperature. The amount of potassium chloride accumulated and the amount of extra carbon dioxide evolved are shown in Fig. 2. Solutions of the same concentration (0·0088 M and 0·0044 M) were added, but the accumulation in these two replicate sets of tissue was less than the accumulation in those used in the previous experiment and both the initial and final rates of accumulation were lower. The carbon dioxide output was increased to a less extent than in the previous experiment and towards the end of the experiment there was an indication of the extra carbon dioxide output decreasing in rate

[1] Concentrations used in these experiments were chosen arbitrarily.

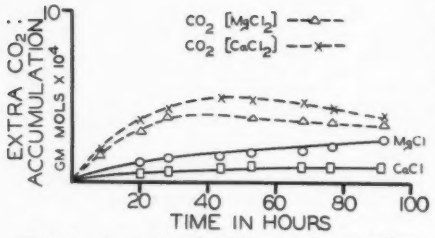

Fig. 4. Amounts of calcium and magnesium chlorides accumulated and of extra carbon dioxide evolved, plotted against time, for tissue placed initially in solutions of 0·0069 M $CaCl_2$ and 0·066 M $MgCl_2$ respectively.

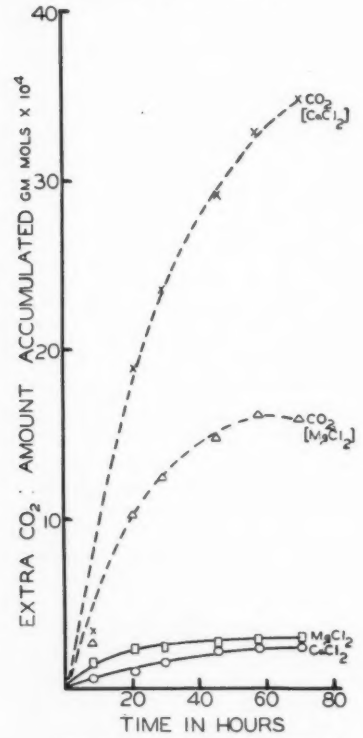

Fig. 5. Amounts of calcium and magnesium chlorides accumulated and of extra carbon dioxide evolved, plotted against time, for tissue placed initially in solutions of 0·0102 M $CaCl_2$ and 0·0056 M $MgCl_2$ respectively.

even in the more concentrated solution, while in the more dilute solution there was a definite decrease in rate at the end of about 50 hours. The difference in accumulation and in respiration between the tissues used in the first and second experiments is discussed later.

Other Monovalent Chlorides.

Another experiment was done using sodium and lithium chlorides. In this experiment (at 23·5° C.) sodium chloride in a concentration of 0·0108 M was added to one set of tissue and lithium chloride in a concentration of 0·0072 M was added to the second set of tissue, the third set being kept in distilled water as a control.

In both treatments respiration increased and the rate was maintained at an approximately steady value through the period of the investigation, the increase in the respiration in the presence of the sodium salt being greater than that in the presence of the lithium salt. The accumulation of electrolyte and the amount of extra carbon dioxide produced are shown in Fig. 3.

Divalent Chlorides.

In two other experiments the electrolytes added to the external solution were magnesium and calcium chlorides. In the first experiment (24° C.) calcium chloride in a concentration of 0·0060 M was added to the external solution of one set of tissue and magnesium chloride in a concentration of 0·0066 M was added to the external solution of the second set of tissue while the third set was maintained as a control.

The results of this experiment differed from those of earlier experiments. There was a slight reduction in the conductivity of the external solutions corresponding to a small uptake of the electrolytes and there was a marked increase in the carbon dioxide output which, however, fell back to the same level as that of the control; indeed in the case of the calcium chloride it seemed to fall below the respiration rate of the control (Fig. 4).

In the second experiment, which was done at a temperature of 25°C., a higher concentration of calcium chloride was used (0·0102 M) and approximately the same concentration of magnesium chloride (0·0056 M). Small quantities of both ions were accumulated over the succeeding period of about 70 hours, but it is striking that the effect of ions upon the respiration was much greater (Fig. 5) than that seen in the preceding experiment. The same rapid fall in carbon dioxide output is to be observed.

DISCUSSION.

The Effect of Electrolyte upon the Respiration.

It is well known that there is a great variation in the ability to accumulate salts shown by tissue from different sources, and tissue which has undergone different treatments after removal from the plant. Sets of tissue examined at different times show great variability in the respiratory activity; this may be associated with the changes in carbohydrate content which have occurred after the tissue has been removed from the growing plant, or with differences in the initial carbohydrate content. The capacity of a tissue to absorb a particular salt varies with the metabolic activity of the tissue and, as pointed out by Stiles and Skelding (1930a), according to the medium in which the tissue has been grown.

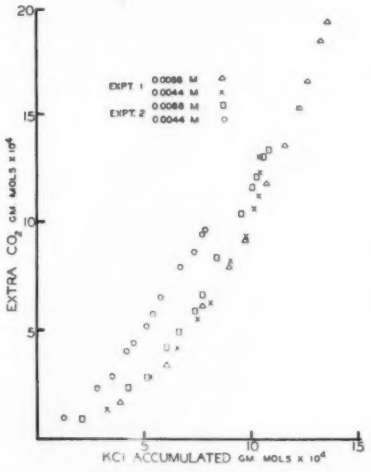

Fig. 6. The relation of salt respiration to salt accumulation in the KCl experiments.

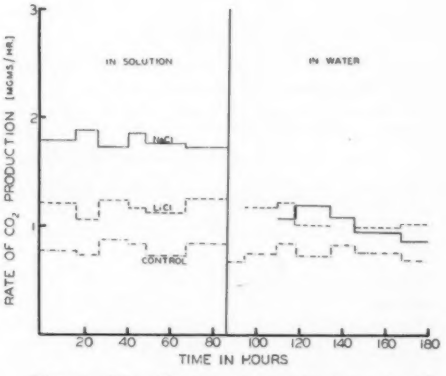

Fig. 7. The effect on the respiration rate of transference from salt solutions to distilled water.

These factors make it desirable that the material should be obtained from a common source and kept under uniform conditions before experimentation. For this series of experiments this was not done and this must be borne in mind when comparing results obtained with one set of tissue with those obtained in another set. For this reason results from replicate sets of tissue are reproduced in the same diagrams. An illustration of the variability in response of two sets of tissue is obtained by comparing both the salt respiration and the salt accumulation in Figs. 1 and 2, where potassium chloride solutions of the same concentration gave markedly different effects on two different sets of tissue.

In spite of the difference in the rate of accumulation from the 0·0088 M potassium chloride in these two experiments, the extra carbon dioxide produced per gm. mol accumulated is approximately the same. This is seen clearly in Fig. 6, where the extra carbon dioxide is shown plotted against the potassium chloride accumulated. As the amount accumulated is increased more carbon dioxide per gm. mol accumulated is evolved. The same effect is shown with the accumulation from the 0·0044 M potassium chloride solution in the first experiment, though in the second experiment the accumulation was accompanied by a rather higher evolution of carbon dioxide during the early stages resulting initially in a somewhat steeper curve. From these results it can be seen that the coefficient $\frac{\text{salt respiration}}{\text{salt accumulation}}$ is constant for accumulation with a certain internal concentration, but increases as the internal concentration increases. The sets of tissue used

in the second experiment had a lower salt respiration (and a lower accumulation rate) than those used in the first experiment. It might at first be thought possible that the extra respiration due to the electrolyte is proportional to the respiratory level prior to the addition of the salt since the respiratory level is often a measure of potentiality for increased metabolic activity. This does not seem to be borne out by these data. Those sets of tissue which had a higher initial respiration gave less salt respiration and less salt accumulation than those which were respiring less actively.

From the two potassium chloride experiments it is to be observed that the tissue subjected to the higher concentration of electrolyte showed, in each case, a greater increase in carbon dioxide production. The initial increase in carbon dioxide production might be expected to be greater in the case of the more concentrated solution if the potassium chloride absorption were not evenly distributed throughout the tissue but were limited by distance from the surface of the disc so that the cells in the centre of the tissue received less electrolyte. Then, the higher concentration of electrolyte would be expected to increase the respiration more since more cells would receive sufficient salt to bring their individual respirations to a maximum. As the rate of accumulation falls, however, differences in the concentration of potassium chloride available to cells at the surface and cells at the centre of the disc would become less marked because the surface cells would be accumulating less rapidly. If that were so a change in the ratio $\frac{\text{extra } CO_2 \text{ in conc. sol.}}{\text{extra } CO_2 \text{ in dil. sol.}}$ would be expected. However the difference in respiration rate is still maintained, the tissue in the more dilute solution continuing to respire less actively than that in the more concentrated solution, suggesting that the difference is not due to lack of homogeneity.

These experiments show clearly that there is an effect of the electrolyte upon respiration and that this effect is proportional to the initial concentration applied externally. This effect persists in time despite a fall in external concentration and increase in internal concentration of electrolyte. This time relationship has never been clear from Lundegårdh's results because he investigated the two factors, respiration and accumulation, which are both dependent upon the external concentration, but did not obtain progress curves for respiration and accumulation. Lundegårdh's original assumption that the increased respiration is due to the ion accumulation is based entirely upon the fact that he found an increase in accumulation was accompanied by an increase in extra carbon dioxide production. Simultaneous increase in these does not necessarily mean that they are related as cause and effect; they may both be effects of a common cause, namely the external concentration of electrolyte.

It has already been pointed out that the carbon dioxide output is increased by the addition of salts to the external solution. This increase is seen in all cases where accumulation takes place. The original conclusion to which Lundegårdh and Burström (1933) were led is that, over the period of their experiment, the total respiration of a plant tissue can be expressed as $R_T = R_G + kA$ where R_T = Total Respiration, R_G = Ground Respiration, kA = Anion Respiration which is a multiple (k) of the total anion absorbed (A).

At first Lundegårdh thought that the anion coefficient, k, was a characteristic for a particular anion and was independent of the cation by which that anion was accompanied. Subsequently, however, he found that this was not universally the case and that the coefficient varied with the nature of the cation. Recently Lundegårdh (1940b) realized, as was pointed out by van Eijk (1939) that the expression should include some factor involving time and, furthermore, Lundegårdh (1940b) says that the expression is valid only for one particular cation and a limited range of concentration and that "the coefficient, k, does not give any evidence of stoichiometrical relation between the amount of absorbed anions and the amount of oxidized glucose. . . . k is an expression of the total work performed in lifting anions from the solution to the cell sap and, accordingly, is dependent upon the manner of translocation and upon the degree of accumulation".

Consideration of Figs. 1 and 2 shows that the increase in respiration due to the presence of salt in the potassium chloride experiment does not bear any stoichiometrical relation to the amount of salt (or anion) accumulated but that the rate of salt accumulation falls off more rapidly than does the extra respiration which may be maintained at a high value throughout the course of the experiment. Consequently the ratio $\frac{\text{salt respiration (in mols)}}{\text{salt accumulation (in mols)}}$ is not a constant but in all

cases shows an increase in time. The effect of adding electrolyte to the external solution is to increase the respiration rate by an amount which is maintained for a considerable time. It does not appear from these results that the respiration rate runs parallel to the number of ions entering the cells during the experimental period or to the rate of accumulation, since the rate of respiration remains steady while the accumulation rate is decreasing. It seems that the presence of salt is capable of increasing the respiration within the first few hours after its contact with the tissue and that this respiratory level is maintained over a wide range of accumulation rates.

The lack of parallelism between the amount of ion accumulated and the amount of extra carbon dioxide evolved makes it necessary to seek another explanation of the relationship between salt respiration and salt accumulation. There is no stoichiometric relationship, as originally suggested by Lundegârdh and therefore the energy relationships of the process and not the chemical equivalents should be considered. The presence of salt in one phase in the cell gives rise to an increase in respiratory activity. That increase in respiratory activity is maintained so long as the electrolytic concentration is maintained in that phase of the cell responsible for respiration—probably a cytoplasmic phase. This behaviour is suggested by the results for the two different concentrations of potassium chloride and the other monovalent chlorides. When divalent chlorides are used the high rate of salt respiration which occurs initially is not maintained during the whole course of accumulation (Figs. 4 and 5).

In order to test the validity of the assumption that the increase in respiration is due to the presence of electrolyte in one particular phase only, it will be necessary to obtain information as to the effect of transferring tissue back to distilled water after considerable amounts of electrolyte are accumulated. On transfer to distilled water most of the accumulated electrolyte concentration is, of course, maintained, the loss of electrolyte to the distilled water which occurs being only a small fraction of that which has been accumulated. In the experiment with sodium and lithium chlorides the tissue was transferred back to distilled water after accumulation had proceeded for about 140 hours. The effect on the rate of carbon dioxide production is shown in Fig. 7, where the respiratory rates for 86 hours prior to the transference to distilled water are given. This suggests that there is, in fact, a decrease after the removal of the external concentration. The same thing is seen in the first potassium chloride experiment in which the external concentration was reduced to a value at which accumulation no longer occurred. Following this cessation of accumulation there was a decrease in the salt respiration, though it did not, in the time of the experiment, return to that of the control.

From these data it is suggested that the entry of ions into one phase of the tissue, probably a cytoplasmic phase, is effective in increasing the respiration. Whether this increase in respiration is due, as Lunegârdh suggests, to an alteration in the oxidation-reduction potentials of the tissue will require further investigation. It is indeed a plausible hypothesis.

The Course of the Accumulation in Time.

It is seen from the figures (1–3) that, in all cases of absorption of electrolyte, the rate of accumulation falls rapidly at first but later reaches a level which changes little over a long period. This is particularly well illustrated in the case of the second potassium chloride experiment (Fig. 2). This change in rate of accumulation has been observed by other workers, both in carrot tissue (Briggs and Robertson, unpublished, and Stiles and Skelding, 1940) and in other tissues (Steward and Harrison, 1939, and Hoagland and Broyer, 1936). No "two-phase course" similar to that observed by Stiles and Skelding (1940a) in carrot tissue was obtained. The curves showed a gradual declining rate of accumulation thereby being more comparable to the chloride absorption curves obtained by Stiles and Skelding (1940b) in the case of manganese chloride. It is probable that the chloride absorption in their manganese experiments is more nearly a measure of accumulation and that the manganese absorption, which is greater, is largely due to exchange.

The relationship of the accumulation rate to time does not seem to have been examined but if, as is generally assumed on the available evidence, accumulated salt remains in solution the concentration ratios and the necessary energy

expenditure will be of primary importance in explaining the form of the curve. In order to accumulate ions within the cell against a concentration gradient energy must be expended and the work applied to the process of accumulation. Since the difference in concentration inside and outside the cell is increasing the rate of accumulation would be expected to fall unless the expenditure of energy increased to meet the increased concentration gradient.

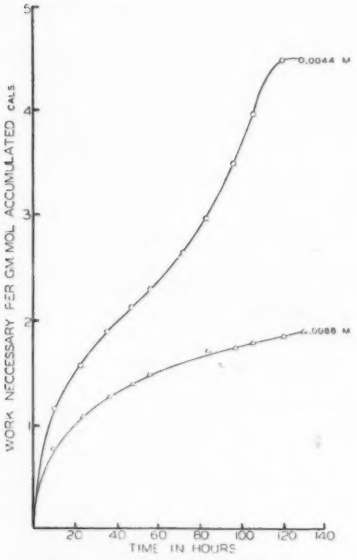

Fig. 8. The osmotic work necessary to transfer 1 gm. mol of KCl from the concentration of the external solution to the concentration within the tissue at different times during the course of the first KCl experiment.

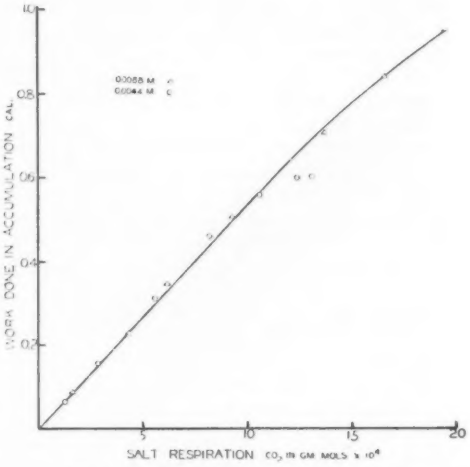

Fig. 9. The relation of work done in accumulation to the salt respiration in the first KCl experiment.

If the external concentration of the salt applied is known and the internal concentration at any given time is calculated, then an estimate of the work to be done in transferring 1 gm. mol from the external solution to the interior of the tissue can be obtained from the formula: $A = RT \log_e \frac{C_i}{C_o}$ where A = Osmotic work in cal. R = Gas constant. T = Absolute temperature. C_i = Concentration within the tissue (corrected for activity). C_o = Concentration in the external solution (corrected for activity).

The osmotic work necessary for the accumulation of 1 gm. mol of the electrolyte at different times during the potassium chloride experiment is shown in Fig. 8. In this calculation C_i is taken as the concentration of salt in the total volume of the tissue. As the ratio $\frac{C_i}{C_o}$ increases the osmotic work necessary for the transfer of 1 gm. mol of electrolyte increases too; more rapidly in the case of the dilute solution than in the case of the more concentrated solution. The accumulation rate in the dilute solution, however, is only slightly less than that of the more concentrated solution.

This suggests that carrot tissue accumulates ions at a rate which is independent of the concentration of the external solution. The tissue, therefore, would appear to do more osmotic work in accumulating from a dilute solution at the lower rate than when it accumulated from a more concentrated solution.

This is seen by comparing the figures in the last two columns of Table 1 which gives the calculated rate of doing work in accumulating from the two concentrations.

TABLE 1.

Time hrs.	Work necessary cal./gm. mol accum. 0·0088 M	0·0044 M	Accumulation rate gm. mols × 10⁴/hr. 0·0088 M	0·0044 M	Rate of working cal. × 10⁴/hr. 0·0088 M	0·0044 M
10·25	768	1,160	0·22	0·21	169·0	243·6
23·67	1,074	1,581	0·14	0·13	150·5	205·9
35·75	1,259	1,900	0·11	0·10	138·4	190·0
47·67	1,378	2,141	0·09	0·08	124·0	171·5
55·67	1,489	2,307	0·08	0·07	119·0	161·6

The same phenomenon is to be observed in the second potassium chloride experiment in which case after the initial period the accumulation rate from the more concentrated solution is very little different from the rate in the more dilute solution. We have, then, an accumulation rate which cannot be increased appreciably by decreasing the osmotic work necessary for a transfer of ions from the external solution but which is dependent upon some internal feature of the tissue.

The tendency for the rate of accumulation to become independent of the external concentration, when this is high, has already been observed by Briggs and Robertson (unpublished) with carrot tissue and is shown in the data of Stiles and Skelding (1940a). In the potassium chloride experiments of Stiles and Skelding it is possible to estimate the average rate of accumulation over the first period if it is assumed that the rate of accumulation is that of the ion which is absorbed in less quantity.

TABLE 2.

Data from Stiles and Skelding (1940a). Absorption of Potassium Chloride Solution.

Data from	Initial concentration.	Period hours.	Absorption gm. mols × 10⁴ K.	Cl.	Average rate accumulation gm. mols/hr. × 10⁴.
Table 3	0·020 M	18·90	5·2	4·4	0·230
	0·005 M	18·67	4·0	4·0	0·210
	0·001 M	18·50	0·88	1·5	0·050
Table 4	0·020 M	20·00	3·3	4·5	0·165
	0·005 M	„	1·5	2·6	0·075
	0·001 M	„	0·2	0·6	0·010

Comparison of the rates of accumulation with the 0·020 M and the 0·005 M solutions indicates that the effect of change of concentration on accumulation rate drops markedly at higher concentration. Hence the rate of accumulation is not greatly dependent upon the ratio $\frac{C_i}{C_o}$. The same phenomenon is illustrated in the results of the investigation on the absorption of manganese chloride (Stiles and Skelding, 1904b, Table 9) in the case of manganese but this is complicated by exchange as well as accumulation. In drawing conclusions as to the form of the relationship between the rate of absorption or accumulation and concentration, consideration must be given to the fact that the lower concentrations of salt will not result in uniformity of concentration at cell surfaces throughout the tissue. The higher the concentration the more nearly will this be the case and it is, of course, only after approximate uniformity of concentration at cell surfaces is reached that further increase in external concentration has a negligible effect on the rate of accumulation.

One simple picture of a process whereby concentration did not markedly affect the accumulation rate would be if there were a system in which one phase of the cell became rapidly fully saturated with the electrolyte and subsequently by another process the electrolyte was withdrawn from this phase into a second phase. This would correspond to an adsorbed film, saturated at low concentration on one side. In such a system if the initial saturation of the first phase were rapid the rate of accumulation as a whole would depend on the rate of withdrawal to the second phase. In other words, the rate of accumulation is dependent upon some factor within the tissue and is not dependent upon the osmotic gradient which must be negotiated between outside and inside solutions.

Much evidence points to the fact that the accumulation of electrolyte in plant cells occurs in the vacuole but it is clear that the electrolyte must first pass into and through the cytoplasm. Whether the electrolyte first saturates the cytoplasm and is then subsequently transferred to the vacuole at a rate which governs the process of accumulation as a whole, can be shown only by subsequent investigation. In this connection the work of S. C. Brooks (1937) is interesting. He found that radio-active potassium ions accumulated in the protoplasm for some time before they entered the vacuole. Further, the changes in potential observed by Lundegårdh (1938) can be attributed to the effect of the cations in replacing hydrogen ions already present in the cytoplasm.

On this picture the fall in the rate of entry of salt in time would be due to the decrease in the rate at which the electrolyte is moved from the first phase where saturation has taken place to the second phase. The decrease in rate may thus be associated with the second phase slowly coming to a point of saturation of electrolyte, energy of course being expended so long as the accumulation to a higher concentration is taking place. The question of how the necessary energy is applied is bound up with the respiratory activity of the tissue during accumulation.

It has already been pointed out that a constant amount of energy, as indicated by carbon dioxide evolution, seems necessary to transfer a given amount of the salt into the tissue at a certain concentration within the tissue. The amount of energy necessary for the transfer is independent of the concentration of the external solution.

There is no *a priori* evidence to assume that the energy which is utilized in accumulation of salt is derived from the extra component of respiration which is induced by the electrolyte. It is, however, a very likely possibility since, presumably, the energy expenditure of the cell which proceeds in distilled water must also proceed in salt solution and energy over and above that necessary for the maintenance of cell processes must be used in accumulation. Lundegårdh (1938) concludes from the results of earlier work that it is the salt respiration which provides the energy for accumulation. This is based on those experiments in which it was shown that the factors which vary the one will also vary the other but do not necessarily influence the ground respiration.

If the evidence is that the energy for the accumulation is being derived from the extra respiration then the rate of accumulation will be a function of both the salt respiration and the increasing internal concentration. Assuming that the osmotic work to be performed depends upon the concentration ratio between an outer phase, e.g. the cytoplasm, and an inner phase, e.g. the cell sap, and that the concentration in the outer phase does not vary over a range of external concentrations then the rate of transfer should be proportional to the energy available, i.e. to the respiration.

A suggestion that this is so can be seen by comparing the initial rates in the potassium chloride experiments:

TABLE 3.

Rate of accumulation gm. mols/hr.	Rate of respiration gm. mols/hr.	Accumulation / Respiration
$6{\cdot}0 \times 10^{-5}$	$1{\cdot}9 \times 10^{-5}$	3·2
$3{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-5}$	3·0
$3{\cdot}7 \times 10^{-5}$	$1{\cdot}2 \times 10^{-5}$	3·1
$2{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-5}$	2·0

It might, therefore, be assumed that the total work is proportional to the concentration ratio, $\frac{C_v}{C_e}$ where C_v is the concentration in the internal phase and C_e is the concentration in the outer phase. C_v increases rapidly as accumulation proceeds and C_e, it is assumed, does not alter over a wide range of external concentration.

As accumulation in a piece of tissue or in a single cell proceeds the work necessary to transfer 1 gm. mol will increase according to the formula: $A' = 2{\cdot}303\ RT \log 10 \frac{C_v}{C_e}$ where

A' = work/gm. mol. accumulated. Since the energy available for accumulation is derived from the respiration and, it is assumed here, from the salt respiration and this energy available does not increase with time, it follows that the rate of accumulation must decrease with time. If this hypothesis is correct and the rate is a function of the internal concentration it will be possible to calculate the concentration C_e, taking the concentration, C_v, as the amount accumulated per volume of tissue. This assumes that the amount of electrolyte in the external phase is small relative to that in the internal phase.

It follows that if C_e is a constant over a period of accumulation, the rates at different times will bear the ratio $\frac{v}{v'} = \frac{\log_{10} \frac{C_v'}{C_e}}{\log_{10} \frac{C_v}{C_e}}$ where v is the rate of accumulation when the internal concentration is C_v and v' is the rate of accumulation when the internal concentration is C_v'. Hence, in the first potassium chloride experiment, after 10 hours, $v = 0{\cdot}22 \times 10^{-4}$ gm. mols/hr., after 50 hours, $v' = 0{\cdot}106 \times 10^{-4}$ gm. mols/hr., $C_v = 3{\cdot}8 \times 10^{-4}$ gm. mols/tissue volume, and $C_v' = 9{\cdot}1 \times 10^{-4}$ gm. mols/tissue volume. C_e, therefore, is $1{\cdot}70 \times 10^{-4}$ gm. mols/14 c.c. = 0·0121 M.

From this value for C_e the change in work necessary for the transfer of 1 gm. mol can be calculated. The concentration values must, of course, be corrected to activities in this calculation. The work necessary for the transfer of 1 gm. mol of potassium chloride is given in Table 4. The total work done in accumulation up to any given time can be obtained by integrating the work necessary for the transfer of one gm. mol and the amount accumulated up to that time.

TABLE 4.

Time (hrs.).	Work/gm. mol (cal.).	Amount accumulated gm. mols × 10⁴.	Total work.
10·3	461	3·90	0·090
23·7	703	6·21	0·227
35·8	821	7·75	0·343
55·7	958	9·55	0·505
83·8	1,064	11·50	0·705
105·8	1,113	12·70	0·840
129·2	1,150	13·65	0·950

If the same value, C_e, for the 0·0044 M potassium chloride solution is taken, the total work can be similarly calculated. These results are given in Table 5.

TABLE 5.

Time (hrs.).	Work/gm. mol (cal.).	Amount accumulated gm. mols × 10⁴.	Total work.
10·3	369	3·3	0·062
23·7	618	5·2	0·156
47·7	824	7·5	0·321
72·2	921	9·1	0·462
96·8	981	10·1	0·558
120·1	997	10·5	0·598
129·2	997	10·5	0·598

If the suggestion that—assuming there is a phase of constant concentration—the energy expenditure on accumulation depends on the energy available from the oxidation in the presence of salt, the total work done will bear a linear relationship to the salt respiration. This is shown in Fig. 9. The relation is linear over the greater part of the course, both in the dilute and more concentrated solution. The more dilute solution showed that there was a departure from the linear relationship when the external concentration reached a low value; in the more concentrated solution there was a suggestion of a slight departure after a considerable time. This might be due to the fact that after a certain amount of salt is accumulated the respiration is maintained at a high level but a lower percentage of this respiration may be available for accumulation; further C_e must fall as the external concentration is very much diminished.

The data of the second potassium chloride experiment can be similarly analysed. Once again it is found that the relationship is linear over the greater part of the range.

The other monovalent chloride results lend themselves to similar analyses but more experimental data are desirable before calculating the relationship of the other chlorides to the respiration and to the accumulatory power of the tissue.

The divalent chlorides investigated differ from the monovalent chlorides, particularly in the fact that the salt respiration returns rapidly to that of the control; it is impossible to say whether the effect of the divalent chlorides in restricting respiration prevents the further accumulation or whether there is some other—perhaps toxic—effect of the divalent cations. The respiration rate of one of the sets of tissue in calcium chloride fell, after some time, to a value less than that of the control. Hence, if the respiration were measured soon after application of the salt it would appear to be increased; if it were measured some time later it would appear to be decreased. This suggests the possibility that the difference between Lundegårdh's results and those of Steward and Preston may be due, not only to difference in material but also difference in duration of experiments.

The Mechanism of Ion Accumulation.

From the evidence outlined in the foregoing section it is suggested that the accumulation of ions which takes place in the living cell is a function of the energy available from the extra respiration which the salt itself induces. This is, in general, in accordance with the theory of Lundegårdh, but no suggestion has been made here that this respiration is particularly an "anion respiration". Lundegårdh's theory of "anion respiration" seems to have been based largely on the fact that he found correlation between amounts of anions entering the cells and the amounts of extra respiration. It seems to the writer, however, that it could equally well be termed a "salt accumulation respiration" since the amounts of anions entering will, in most circumstances, be a measure of the amount of salt accumulated since most of the exchange which occurs in normal plant cells seems to be a cationic exchange and very little is anionic. It is likely that the cations from the external solution can enter freely into the cell whereas the anions cannot penetrate the cytoplasm owing to indiffusible organic anions such as the proteins on the acid side of their isoelectric points. Diffusion potential measurements across carrot tissue (Briggs and Robertson, unpublished) indicate that there is less resistance to the movement of cations than to anions. It is, therefore, possible that the entry of the cations would have the effect postulated by Lundegårdh (1940a) on the respiration. Lundegårdh suggests that the presence of metallic cations replacing hydrogen ions increases the oxidizing power of the cell in the region where respiratory oxidations are taking place. That this increase in respiration would bring about the entry of the salt into the vacuoles of the cells is not self evident and the only evidence that this is the case is that obtained by Lundegårdh and Burström (1936) that those factors which tend to promote or inhibit ion accumulation tend to promote or inhibit salt respiration.

This general theory of Lundegårdh's has been criticized by Hoagland and Steward (1940) on the grounds that the sharp distinction in the relation of the metabolism to the uptake of anions and cations postulated by Lundegårdh is not borne out by general experience. In the view of the writer general evidence, such as that obtained by Broyer and Overstreet (1940), does seem to point to the fact that cations can enter readily by exchange involving no expenditure of energy on the part of the cell. On the other hand the ionic accumulation in which both cations and anions are involved does necessitate expenditure of energy by metabolic processes. At the present state of our knowledge there is not sufficient evidence to dissociate the effects of cation and anion upon respiration. The early claim made by Lundegårdh that the anion effect on respiration was independent of the entry of the cation was later withdrawn in the light of his subsequent work. Recently Lundegårdh's theories have been criticized by Lipman (1940) who says that "Lundegårdh's views on the role of anions are nothing less than an over emphasis on one aspect of the whole complex subject of ion absorption". This criticism does not seem to be justified if what Lundegårdh originally termed anion respiration is taken as being the respiration associated with salt accumulation since, as Lundegårdh himself pointed out, it is not independent of the entry of the cation accompanying the anion.

Criticism of Lundegårdh's theory expressed in the recent paper of Steward and Preston (1941) fails to recognize completely the modifications which Lundegårdh (1940b) has made in his original suggestions. Further, as has already been pointed out (Robertson, 1940), Lundegårdh's conclusions as to the effect of potassium and calcium salts on wheat tissue are not invalidated by similar experiments on potato tissue where different effects are obtained. The

experiments quoted in this paper show that, in behaviour to calcium chloride, carrot tissue seems to resemble the tissue used by Lundegårdh in giving an increase in respiration immediately after the application of the salt, but resembles that used by Steward and Preston in giving a subsequent decrease in respiration rate below that of the control.

The work of Steward and Preston on potato tissue suggests very strongly that, in that case, the accumulatory mechanism is associated in some way with the nitrogen metabolism and that the component of respiration which is linked to protein synthesis seems to be closely associated with salt accumulation. So far, no investigations on carrot tissue have been made to determine whether the salt respiration is associated with oxidation processes in carbohydrate respiration, as was suggested by Lundegårdh, or with the protein synthetic mechanism, as Steward suggests. It must be remembered, however, that either system would, as Steward and Preston point out, result in a liberation of energy and only future work will show whether, in carrot tissue, salt accumulation effects are due entirely to energy derived from some oxidation process or may be partially dependent on some accompanying process such as protein synthesis.

In this paper it has been pointed out that there is a close relationship between the rate of accumulation and the osmotic work to be done in accumulating the electrolyte if it is assumed that there is a phase in which concentration is maintained independent of a wide range of external concentration. There is an effect of the electrolyte on the respiration and it is possible that this extra respiration is responsible for the ionic accumulation. It might be pointed out that, assuming that hexose sugar is respired, the extra respiration liberates, per unit time, very many times as much energy as is expended in the accumulation process, calculated on the basis of the work being done against the osmotic gradient. For instance in the first potassium chloride experiment, the total amount of carbon dioxide produced was $19{\cdot}48 \times 10^{-4}$ gm. mols. Assuming that the liberation of each gm. mol of carbon dioxide is accompanied by the release of 112,333 cal., the total energy released in salt respiration would be 219 cal. During the same period the total work done in accumulation is 0·95 cal.

Though Lundegårdh adduced evidence for a difference in nature of the ion respiration and the ground respiration, the possibility remains that in other types of tissue, such as carrot tissue, the salt respiration is merely an increase in the normal rate of the normal respiration process due to an effect of the ions upon some particular reaction in the process or is an effect on some subsidiary process such as nitrogen metabolism.

SUMMARY.

The effect of the accumulation of chlorides upon the respiration of cut carrot tissue has been investigated. The accumulation was determined by measuring the change in conductivity of the solution in contact with the tissue; the respiration was determined by measuring the carbon dioxide evolution.

All five chlorides investigated caused an initial increase in respiration rate above the rate of control tissue kept in distilled water. In the monovalent chlorides, this high respiration rate was maintained in time while the rate of salt accumulation decreased. In the divalent chlorides, the respiration, although initially above that of the control, fell to the same value as the control and in one case fell below the level of the control.

The relationship of the amount of salt accumulated to the amount of extra carbon dioxide produced in salt respiration is discussed. In the case of potassium chloride, in spite of differences in the rate of accumulation in different sets of tissue, the extra carbon dioxide produced per gm. mol of salt accumulated was approximately the same, increasing similarly with time in all cases.

The course of accumulation in time is discussed in relation to the energy requirement of the process. It is shown that the concentration difference between the external solution and the tissue does not seem to govern the rate of the process at higher concentrations. A suggested interpretation is that there is an outer phase in the cell of constant concentration of salt and that the salt is removed into a second phase by an energy-expending process. Since the energy available as indicated by the respiration rate is approximately constant, the possibility that the falling rate is due to the increase in concentration between the outer phase and inner phase is explored. On the assumption that the outer phase retains a constant concentration, the total work done in accumulation is shown to bear a linear relation to the total salt respiration over the greater part of the range.

The relation of these results to other recent work on salt accumulation is discussed.

Acknowledgments. The writer wishes to express his thanks to Professor Eric Ashby, in whose department this work was done, for his interest and helpful criticism of the manuscript, and also to Dr. T. Iredale of the Chemistry School, University of Sydney, for helpful criticism of the manuscript.

REFERENCES.

Briggs, G. E. and Robertson, R. N., unpublished data; work done at Botany School, Cambridge, 1937–8.
Brooks, S. C. (1937): Trans. Faraday Soc., 33, p. 1002.
Broyer, T. C. and Overstreet, R. (1940): Amer. J. Bot., 27, p. 425.
van Eijk, M. (1939): Rec. Trav. Botan. Neerland., 36, p. 561.
Hoagland, D. R. and Broyer, T. C. (1936): Plant Physiol., 11, p. 471.
Hoagland, D. R. and Steward, F. C. (1939): Nature, 143, p. 1031.
Hoagland, D. R. and Steward, F. C. (1940): *Ibid.*, 145, p. 116.
Lipman, C. B. (1940): Ann. Rev. Biochem., 9, p. 491.
Lundegårdh, H. (1939); Nature, 143, p. 203.
Lundegårdh, H. (1940a): *Ibid.*, 145, p. 114.
Lundegårdh, H. (1940b): Ann. Ag. Coll. Sweden, 8, p. 234.
Lundegårdh, H. and Burström, H. (1933): Biochem. Z., 261, p. 235.
Lundegårdh, H. and Burström, H. (1935): *Ibid.*, 277, p. 223.
Robertson, R. N. 1940): Nature, 145, p. 937.
Steward, F. C. (1932): Protoplasma, 15, p. 29.
Steward, F. C. (1937): Trans. Faraday Soc., 33, p. 1006.
Steward, F. C. and Harrison, J. A. (1939): Ann. Bot., N.S., 3, p. 427.
Steward, F. C. and Preston, C. (1941): Plant Physiol., 16, p. 85.
Steward, F. C., Stout, P. R. and Preston, C. (1940): Plant Physiol., 15, p. 409.
Stiles, W. and Skelding, A. D. (1940a): Ann. Bot., N.S., 4, p. 329.
Stiles, W. and Skelding, A. D. (1940b): *Ibid.*, 4, p. 673.

A SIMPLE WEIGHT-BURETTE FOR USE IN ORGANIC ANALYSIS

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The determination of the molecular weight of alcohols of the steroid group is one of the means by which a compound can be allocated to a particular series. Many sterols have a molecular weight in the vicinity of 400 and differ in structure only by a single CH_2 group in the side chain. Therefore it is essential to have a method capable of detecting such small differences. Physical methods based upon elevation of boiling point or depression of freezing point are not sufficiently accurate. Furthermore, the micro method of Rast is often inapplicable because the high temperature required to melt camphor may lead to destruction of the substance under investigation. The figures for carbon and hydrogen by combustion are not dependable. Thus with cholesterol $C_{27}H_{45}OH$ (M.W. = 386·4) and a plant sterol $C_{29}H_{49}OH$ (M.W. = 414·4) the difference in carbon is only 0·13 p.c., and for hydrogen 0·15 p.c., figures which represent the error inherent in a combustion analysis. Free sterols following crystallization often retain solvent which cannot always be removed without alteration of the substance. This retention of solvent, will of course, further complicate the identification. The plant sterols illustrate these difficulties. Thus stigmasterol a compound found in certain vegetable oils, was, on the basis of combustion figures, thought to have the composition $C_{30}H_{50}O$ (Windaus and Hauth, 1906); sitosterol, on the other hand, was believed to have the composition $C_{27}H_{46}O$ (Burian, 1897). However, Sandqvist and Gorton (1930) showed definitely that stigmasteryl-acetate has a molecular weight of 454·4 and therefore the composition of the free alcohol is $C_{29}H_{48}O$. Sandqvist and Bengtsson 1931) obtained values for sitosteryl-acetate which agree only with the formula $C_{29}H_{50}O$ for sitosterol. These results were obtained by quantitative saponification of the sterol acetates. 0·4–0·5 gm. of the acetate and 30 ml. N/10 alcoholic potash were taken and saponified for an hour. Since the back titration with standard acid can be carried out with great accuracy, the saponification method is regarded as the most satisfactory for the decision of the molecular or rather equivalent weight of an ester whose components are alkali stable. In the course of a recent investigation at this Institute, it was desirable to determine the molecular weight of the alcoholic fraction of the non-saponifiable matter of mussels. For the reasons given above the technique of Sandqvist and Gorton was chosen. Only small amounts of materials were available and with quantities of the order of 0·1 gm. of the sterol acetate, the difference in the volumes of N/10 KOH necessary to saponify cholesteryl acetate and ergosteryl acetate is 0·05 ml. so that the analysis must be accurate to at least 0·02 ml. in order to give an unambiguous result. Using a 5 ml. micro burette of the usual type, the titres are subject to errors in drainage and in the reading of the meniscus. These objections may be overcome by a weight burette, the use of which involves no more skill than weighing to a few milligrams on an analytical balance. With the greatest precautions, such a precision cannot be obtained with a volumetric burette. In addition, a weight burette needs no calibration. The usual type has shortcomings which have been overcome in the design in Fig. 1.

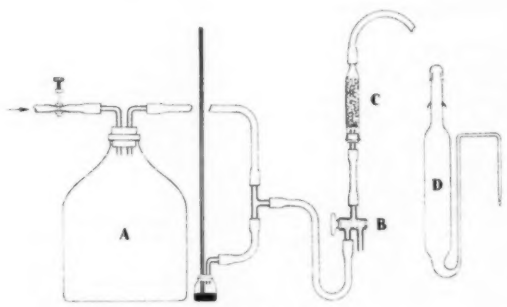

Fig. 1.

A. Pressure bottle connected to mercury manometer.
B. Two-way tap.
C. Soda-lime tube.
D. Weight burette drawn to quarter natural size.

The outlet is drawn out to a capillary which will deliver drops weighing about 10 mg. Such a burette when filled does not lose weight by evaporation during the necessary manipulations, nor is

there any loss of liquid from the capillary when the liquid in the main chamber is at a higher level than in the side arm. Burettes with still smaller drops can be readily made, but with drops weighing 5 mg. the titration time would be unduly prolonged and no great advantage gained. The burette is operated by air which is compressed in a 4 l. glass bottle by a bicycle pump. The pressure at the beginning of the titration is about 20 cm. mercury and towards the end is decreased to 2·5 cm. in order to obtain better drop control. At the end of the titration the two way tap (B) is turned so as to shut off the pressure bottle from the burette and also to expel compressed air from the latter into the atmosphere. The analysis is carried out in the following manner. 80 to 100 mg. of an ester having an assumed equivalent weight of 400 to 500 are weighed into a test tube of resistant glass, 5 ml. N/10 alcoholic KOH are added and the walls rinsed with 0·5 ml. of neutral absolute alcohol. The tube is placed in a hot box, a Hopkin's condenser is inserted, and the contents heated for from 1 to 1½ hours.[1] After cooling to room temperature the condenser is lifted and rinsed with 0·5 ml. of absolute alcohol. 0·05 ml. of the achromatic indicator of E. L. Smith (1930) is added[2] and the solution titrated with N/10 HCl from the weight burette in the usual manner.

A blank on the alcoholic KOH must be carried out omitting the substance under investigation. The following analyses demonstrate the suitability of the method for determining equivalent weights of esters:

			Molecular weight found.	Molecular weight calculated.
0·1396 gm. Cholesteryl acetate	consumed	3·257 ml. N/10 KOH	428·6	428·4
0·1010 gm. „ „	„	2·367 „ „ „	426·6	
0·0812 gm. Ergosteryl acetate	„	1·858 „ „ „	437	438·4
0·1011 gm. „ „	„	2·32 „ „ „	436	
(1 hour saponification for above 4 estimations)				
0·137 gm. Cholesteryl benzoate	„	2·84 „ „ „	482·4	490·7
(1½ hours saponification)				
0·0933 gm. „ „	„	1·93 „ „ „	484	

[1] These times have been found satisfactory for cholesteryl acetate and ergosteryl acetate, but if other esters are used the saponification times must be determined.

[2] The indicator has the following composition: 0·02 gm. methyl red, 0·01 gm. methylene blue, 1·0 gm. phenolphthalein, 100 ml. 95 p.c. alcohol.

SUMMARY.

A tap-less weight-burette is described. It has been used in the estimation of the equivalent weight of sterol esters by the saponification method.

Acknowledgment. The author desires to thank Mr. A. Doutch for his technical assistance.

REFERENCES.

Burian (1897): Monatsh. der Chemie, 18, p. 551.
Sandqvist, H. and Bengtsson (1931): Ber. d. deutsche chem. ges., 64, p. 2167.
Sandqvist, H. and Gorton, J. (1930): *Ibid.*, 63, p. 1935.
Smith, E. L. (1930): Quart. J. Pharm., 3, p. 499.
Windaus, A. and Hauth, A. (1906): Ber. d. deutsche chem. ges., 39, p. 4378.

INFLUENZA VIRUS "A" INFECTIONS OF CYNOMOLGUS MONKEYS

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There is strikingly little information in the literature on experimental infections with influenza viruses in primates other than man. Apart from experiments on chimpanzees reported by Long and Bliss (1931) and by Dochez in commenting on their paper I have been able to find no reports on the subject.

The experiments to be described show that the common Malayan monkey usually referred to as *Macacus cynomolgus* but more correctly as *M. irus* can be infected with several strains of virus. Symptoms are usually trivial, but with one strain extensive consolidation was produced with almost complete regularity. In several instances the infection terminated fatally. All the strains of influenza virus tested induced infection as judged by the appearance of circulating antibody in the inoculated monkeys.

TECHNIQUE.

Most of the monkeys were young specimens of *M. irus* weighing 1–2 kg. and with their permanent dentition incomplete. A few were larger animals up to 4 kg. with permanent canines but no influence of age on the type of response could be recognized. The standard method of inoculation was intratracheal. Under ether anaesthesia an incision about 2 cm. in length was made through the skin and fascia, the muscles were separated and the trachea exposed. A dose of 1–1·5 c.c. depending on the size of the monkey was injected downwards into the tracheal lumen and the animal immediately raised to a vertical position to allow the fluid to run down into the bronchi. The incision was then closed with a single suture. For intranasal inoculations 1 c.c. of fluid was dropped with a pipette into the nostrils with the monkey partly inverted and lightly anaesthetized. Rectal temperatures were taken twice daily for four days after inoculation.

The antibody response to inoculation was determined by titration of serum taken fourteen days later. Blood samples were obtained from the saphenous vein.

In those animals which died or were killed for examination within a few days of inoculation, material for titration and passage was obtained by washing out the bronchial tree. A cannula was tied into the trachea and a syringe containing 10–12 c.c. of broth attached. This volume was injected and then as much as possible drawn back into the syringe usually about 5–7 c.c. This fluid was cultured on blood agar and filtered through a gradocol membrane (A.P.D. about 0·9 μ) for titration and amniotic inoculation.

The material used for inoculation was normally the pooled allantoic and amniotic fluids from chick embryos inoculated two (sometimes three) days previously by the amniotic route with the strain required. The virus content of these fluids was titrated by whatever method was most appropriate for the strain in question. The chorioallantoic method was used for the strain "W.S. egg" with which the largest number of animals were inoculated.

The strains used included two recent locally-isolated strains McD. 1940 (Burnet and Foley 1941) and R. 1939 (Burnet and Lush, 1940), the mouse strain "W.S.M." and two strains fully adapted to the chorioallantois "Melbourne egg" and "W.S. egg".

Antibody titrations were made by the chorioallantoic method against the strains "Melbourne egg" and "W.S. egg". A few confirmatory tests mere made in mice with the appropriate mouse-adapted strains "Melbourne", "W.S." and "Swine".

In the course of the work on the more severe infections with the monkey passage strain "W.S. egg" a considerable number of radiographs were made through the co-operation of Dr. Barbara G. Wood, resident radiologist of the Royal Melbourne Hospital.

The Response to Infection.

Three monkeys inoculated intranasally with strain "R" and two with "W.S. egg" showed no symptoms or temperature rise. After intratracheal inoculation there was usually a definite

[1] Work carried out with the aid of grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

temperature rise within 24 hours. This often persisted into the second day after inoculation and on one or two occasions into the third. The standard temperature of these monkeys varies from animal to animal between 100° and 102° F. In Fig. 1 there are shown two series of temperature responses in previously normal monkeys inoculated respectively with "R" and "W.S. egg" strains. These results may be taken as typical of those obtained throughout

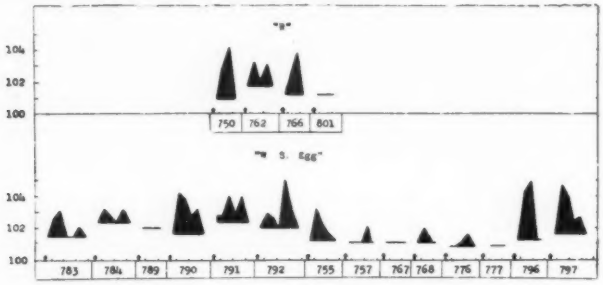

Fig. 1. The temperature response to intratracheal inoculation of influenza virus.

Two series of monkeys are shown, four inoculated with strain "R", and fourteen with "W.S. egg". The temperatures for the two or three days following inoculation are shown, the area darkened representing significant rise above the animal's normal level.

the experiments. It will be seen that of the eighteen monkeys considered four failed to show any rise, five showed a rise on the first day only and three showed a rise beginning on the second day, while the remaining six had a raised temperature for both the first and second days after inoculation. In monkeys dying with pneumonia the temperature fell to subnormal levels on the third or fourth day and remained low till death. Sick monkeys showed no obvious respiratory symptoms, they were inactive and refused to eat, sitting huddled up with roughened fur and apathetic expressions.

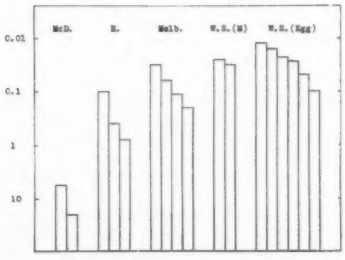

Fig. 2. The antibody response fourteen days after a primary intratracheal infection of monkeys inoculated with strains "McD.", 1940; "R", 1939; "Melbourne", 1935; "W.S." (mouse) 1933; "W.S." (egg) 1933.

The titrations were made against "Melbourne egg" or "W.S. egg" by the chorioallantoic method and the values are expressed with the usual inverted logarithmic scale showing the percentage to which the focal count is reduced.

Inoculation of uninfected embryonic fluids intratracheally produced no temperature change whatever in two monkeys and in twelve re-inoculations with virulent material in monkeys previously infected, only one monkey showed any significant rise. This animal (No. 717) will be discussed later. This is adequate evidence that the temperature responses observed are specific results of infection with the virus of influenza.

The Antibody Response to Primary Tracheal Infections.

All surviving monkeys were bled fourteen days after infection and the serum tested on the chorioallantois for its inactivating power on "Melbourne egg" virus or in the case of monkeys inoculated with "W.S." strains against the corresponding egg virus. The technique has been previously described (Burnet, 1936; Burnet, Keogh and Lush, 1937). The results are expressed as the percentage of "surviving" foci obtained when undiluted serum is mixed with an appropriate concentration of virus and after two hours contact at refrigerator temperature, tested on the chorioallantois of twelve-day chick embryos.

Fig. 2 shows the antibody titres obtained after infection with those strains which were used to inoculate two or more monkeys. It will be seen that there is a considerable regularity in the level to which the antibody titre rises for any one strain and that there are distinct differences in the antigenic power of different strains, "W.S." strains being the most effective.

The Antibody Responses to Repeated Intratracheal Inoculations.

Two monkeys, 706 and 717, were given four successive inoculations with intervals of about two months between each inoculation. The strains used were alternately of "Melbourne" and "W.S." type, the first inoculation intranasal, all subsequent ones intratracheal. Bleedings were made at the time of and fourteen days after each inoculation. Serum titrations showed a sharp rise in antibody after each inoculation except for a final inoculation of M706. Fig. 3 shows the changes in titre of serum from M717 against "Melbourne" and "W.S." egg strains. The first three inoculations produced a series of step-like rises of the "Melbourne" titre; then there was a fall and very little rise in response to the final "W.S." inoculation. This last however has a definite effect on the "W.S." titre increasing it more than twenty fold. The early stages of M706 were very similar but there was no detectable response to the fourth inoculation ("W.S.") the final "W.S." titre being only one-tenth that shown by the fourth bleeding. No detailed experiments on complement fixation were carried out but it may be noted that pooled serum from the final bleeding of 706 and 717 has been used by Miss Foley as a high titre standard serum with which to assess the value of various egg fluids as complement-fixing antigens. This work will be reported in another paper.

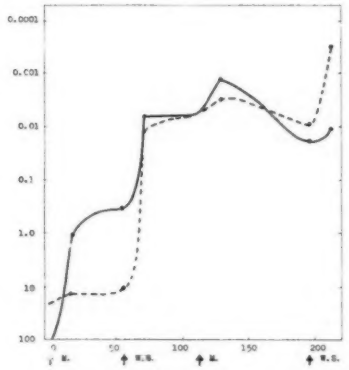

Fig. 3. Antibody changes in Monkey 717 given alternate inoculations of "Melbourne" and "W.S." type strains.

Solid line antibody titre against "Melbourne egg"; broken line against "W.S. egg".

Time in days. Antibody shown as percentage to which the focal count is reduced by undiluted serum. Higher values calculated according to the method described in Burnet, Keogh and Lush (1937). Reversed logarithmic scale.

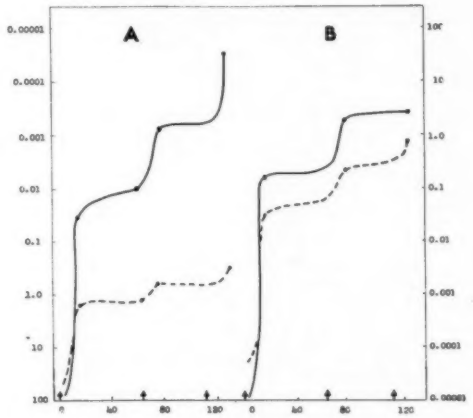

Fig. 4. Antibody changes in Monkey 783 given three intratracheal injections of "W.S. egg" strain.

A. Chorioallantoic titrations against "W.S." solid line and "Melbourne" broken line.

B. Mouse titrations against "W.S." solid line and "Melbourne" broken line.

In A the same conventions are used as in Fig. 3. In B the antibody titre is expressed in terms of two immune ferret sera arbitrarily taken as standards for "Melbourne" and "W.S." antibody.

Time in days.

A second pair of monkeys, 783 and 784 were given repeated intratracheal injections of the same type ("W.S.") and their serological responses studied in detail. The findings for M783 are shown in Fig. 4 which indicates the changes in the virus-inactivating power of the serum as tested against "Melbourne" and "W.S." strains both in mice and on the chorioallantois. M784 showed similar changes after the first and second inoculations; it was accidentally killed before a third dose was given.

Examination of Fig. 4 shows again a well marked antibody response to each intratracheal inoculation, the third rise being much more distinctly visible with chorioallantoic titration than by the mouse lung method. The most striking feature of the results is the extreme specificity of

chorioallantoic titrations as compared with mouse titrations. The final (sixth) bleeding is a little over 10,000 times as active against "W.S. egg" as against "Melbourne egg", yet in mice the difference is only about three fold in favour of the "W.S." strain. The same features were shown in M784 whose fourth bleeding showed a ratio of about 1: 1,200 between "Melbourne" and "W.S." titres on the chorioallantois but nearly equal activity in mice.

A monkey receiving two "Melbourne" type inoculations (M749) showed considerably less specific responses, the chorioallantoic titres being 0·0004 p.c. against "Melbourne" egg and 0·016 p.c. against "W.S. egg" (40: 1) while the corresponding ratio in mouse titrations was was about 8: 1.

The Appearance of Antibody to Swine Influenza Virus in Monkeys Given Repeated Intratracheal Inoculations.

The successive bleedings from Monkeys 706 and 717 were tested for the presence of swine influenza antibody by the usual technique in mice against a mouse substrain of Shope's Swine 15 virus.

Table 1 gives the results with serum 717. Controls not shown in the table included direct titration of the stock virus used and test with dilutions of standard immune ferret serum F56. Following the Hampstead usage the titre is expressed as a fraction of the titre of the ferret immune serum. Serum from M706 behaved differently, the successive titres being 2) 0·00004, 4) 0·004, 6) 0·004, 8) 0·002, i.e. there is only an insignificant increase.

TABLE 1.

Titration of successive serum samples from M717 for swine antibody.

Serum.	S/25 + V	S/5 + V	S + V	S + V/10	S + V/100	S + V/1,000	Titre.
717 (2)					45 46 46	3 3 2	0·00004
717 (4)				2 2 1	0 0 0		0·008
717 (6)	45 47 47	2 1 1	1 0 0	0 0 0			0·4
717 (8)	47 47 2	1 1 0					0·8

Titre expressed in terms of that of serum F56 from a ferret convalescent after Swine influenza virus infection.

The serum virus mixtures used are indicated in the form: S/25 + V — equal volumes of serum diluted 1: 25 and undiluted stock virus, and so on.

Mouse lesions are indicated by the usual symbols: 45 — death with complete consolidation on the 5th day. 3, 2, 1 — survival for seven days with diminishing amounts of consolidation. 0 — no lesions.

Except for the first intranasal inoculation when two different substrains of "R" were used, one for each animal, all the inoculations of 706 and 717 were identical. It is noteworthy however that M706 showed no temperature rise with any inoculation while M717 gave temperature responses of non-immune type after the second and third inoculations but none after the fourth. There is no possibility that M717 was accidentally inoculated with swine virus as this strain was never inoculated into chick embryos during the period of these experiments. The failure of M706 to produce swine antibody in itself provides a valid control since if any of the inocula had contained swine virus both animals should have shown the response.

A similar development of swine type antibody was shown by Monkey 783 whose serological responses to three successive inoculations with "W.S." are shown in Fig. 4. The final serum tested against "Swine" virus in mice in parallel with the standard ferret serum F56 gave results indicating a titre of approximately 0·2 in terms of the standard. This is close to the value shown with the later bleedings of M717.

A Series of Fatal Infections Produced by Strain "W.S. Egg".

Two monkeys, M787 and 788, were inoculated intratracheally with a mixture of the two fully adapted egg strains "Melbourne" and "W.S.". Both showed the usual type of temperature response on the first and second days after inoculation. M787 showed no further symptoms but on the third day M788 had a subnormal temperature (99·4° F.) and appeared sick. It became gradually weaker and died eight days after inoculation. Post mortem examination showed abnormalities only in the thorax. The pleural cavities were filled with almost transparent fibrin clot and there was similar fibrinous exudate in the loose mediastinal tissues. The lungs showed large areas of pale pink consolidation in the lower lobes and smaller areas in the other lobes. On section the lungs were highly oedematous but except for the areas of consolidation at the bases were air-containing throughout. Much frothy fluid exuded from the cut surface which was irregularly mottled with pinkish and yellow areas, the latter being mostly centred around

bronchi. Smears and cultures gave no evidence of bacterial infection and attempts were made to re-isolate the virus from the lung tissue. Apparently very little virus remained as embryos inoculated amniotically remained healthy and only one mouse showed a small area of consolidation. From this mouse a typical influenza virus was recovered which was easily recognized as "W.S. egg" by its power to produce chorioallantoic foci and death with haemorrhagic encephalitis in chick embryos and active lesions in mice. Subsequent serological tests confirmed the identification. The re-isolated virus grown amniotically was inoculated intratracheally into two monkeys (789 and 790). The first showed no temperature response or symptoms. M790 had an active first and second day rise reaching 104·2° F. It was killed on the third day and showed patchy areas of consolidation in the lower lobes particularly on the left side. Tracheal washings were filtered through sterile filter paper and a gradocol membrane and titrated on the chorioallantois. Only an average of 10·5 foci was obtained from 0·05 c.c. amounts of undiluted filtrate. Cultures from the original washings remained sterile. The strain isolated from M790 after one further amniotic passage was inoculated intratracheally into M791 and M792. Both showed temperature responses and M791 became sick on the fourth day and died early on the sixth day after inoculation. M792 was killed for examination 66 hours after inoculation. M791 on post-mortem examination showed pale consolidation involving almost the whole of the lungs except for the peripheral zones of the two upper and right middle lobes. There was a little free fluid in the right pleural cavity and some tags of fibrin along the lower edge of the lung. Tracheal washings again gave only a small amount of virus averaging twelve foci with undiluted filtrate. M792, killed at three days, showed extensive central consolidation of the lower lobes reaching the lung surface at several areas particularly on the left side. Tracheal fluid was sterile on culture and gave an average of two foci when tested undiluted. The next passage was made with fluids from an embryo inoculated amniotically with the filtrate from M792. As before one animal was left to die (M755) and the other M757 was killed 48 hours after inoculation. Titration of the tracheal fluid filtrates from these monkeys showed higher values. M757 killed at two days gave 3×10^4 foci per 0·05 c.c. and M755 dead 90 hours after inoculation gave 90 foci. The post-mortem findings were similar to those of the previous passage. It may be noted that the lung of M755 was very oedematous and in the course of washing out the tracheobronchial system more fluid was recovered than was injected. The next two passages were associated with less striking lesions and owing to shortage of animals the series was not continued further. The results are summarized in Table 2.

TABLE 2.

Results of intratracheal inoculation of monkey passage strain of "W.S. egg" influenza virus.

Monkey No.	Inoculum.	Intervening passages.	Result.	Amount of virus isolated from tracheal washings.	Lung lesions.
788	W.S.E. + M.E.		T † 8d	Trace	+++
		M, Am. Am.			
789	W.S.E. (M788)		N S		
790	,,		T K 3d	$2{\cdot}1 \times 10^2$	++
		Am. Am.			
791	W.S.E. (M790)		T † 5½d	$2{\cdot}4 \times 10^2$	+++
792	,,		T K 3d	40	++
		Am.			
755	W.S.E. (M792)		T † 4d	$1{\cdot}8 \times 10^3$	+++
757	,,		T K 2d	6×10^5	++
		Am.			
767	W.S.E. (M757)		N K 2d	4×10^4	+
768	,,		T S		±(X ray)
		Am.			
776	W.S.E. (M767)		N K 3d	33	++
777	,,		N S		−(X ray)

Abbreviations: M = mouse. T = significant temperature rise. S = survived. Am = amniotic. = no significant temperature response. K = killed on day shown.

Titres of filtrates from tracheal washings shown as foci produced per c.c. Lung lesions are indicated by plus signs according to the extent of consolidation found either post-mortem or by radiography.

It is unfortunate that circumstances made it impossible to elucidate the reasons for the irregularities in the series. The striking effect of inoculation during the third and fourth passages suggested that a rapid increase in virulence for the monkey had occurred but the relative failure of the next two passages made that seem less probable. Possibilities that would need consideration in future work are that the intensity of the lesions produced may depend on minor

variations in the inoculum such as e.g. the viscosity of the embryo fluids or on physiological conditions in the monkeys as modified e.g. by environmental temperature. It will be observed that virus was re-isolated in largest amount from monkeys killed at two days after inoculation and that in the three monkeys dying of infection the amount recovered was inversely related to the length of survival. In view of the early temperature rise and the fact that histological changes are well developed in two days it seems very probable that even larger amounts of virus would be obtained 24 hours after inoculation.

Histology.

The main features of the lesions when examined two to three days after inoculation were acute bronchiolitis, interstitial pneumonia and exudation of coagulable fluid with relatively few cells into alveoli.

Sections from M757 killed two days after inoculation, were made through the bifurcation of the trachea and through the right lower lobe in a region showing typical consolidation. Nothing abnormal was observed in the epithelium of the main bronchi and in this as in all other sections examined the intensity of the damage to the respiratory epithelium increased as the terminal bronchioles were approached. In a medium-sized bronchus localized areas of epithelium were affected, the process having the appearance of a coagulation necrosis of the most superficial ciliated cells with proliferation, disorganization and leucocytic infiltration in the deeper layers. Bronchioles showed practically complete desquamation of epithelium, only a few disorganized cells resting on the smooth muscle layer. There was extensive cellular accumulation around the bronchioles about one-third of the cells being polymorphonuclear leucocytes. Alveolar change was most marked in the neighbourhood of affected bronchioles. The alveolar walls were thickened and cellular and the alveoli contained coagulated fluid with a few polymorphs and alveolar cells: here and there were alveoli containing blood but this was always a minor feature.

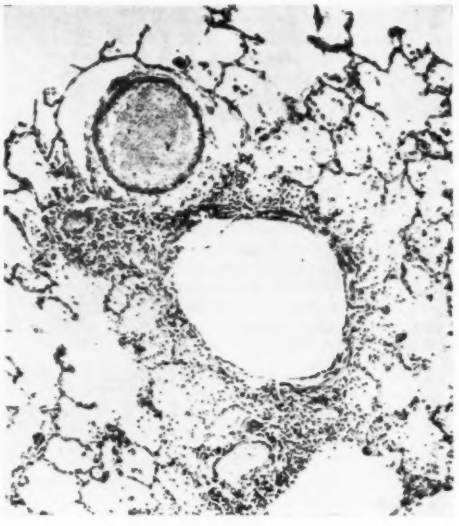

Fig. 5. Monkey 767 killed two days after intratracheal inoculation. Low power view of bronchiole showing loss of epithelial lining, peribronchiolitis and minimal involvement of alveoli. × 85.

Sections from M755 given the same inoculum as M757 and dying of pneumonia after four days showed a continuation of the same process. The large bronchi were normal and there was progressively more damage as the bronchial divisions became smaller. Practically all alveoli in the consolidated regions were filled with a fibrinous coagulum containing moderate numbers of cells mainly mononuclear (see Fig. 6). The fibrinous coagulum also filled many of the terminal bronchioles but there was no indication that plugging of the smaller bronchioles with desquamated cells and leucocytes played any part in producing the consolidation.

The healing process could be seen in action in sections from Monkeys 793 and 797 killed seven and ten days after inoculation. These sections showed clearly the essentially interstitial character of the lesion, greatly thickened alveolar walls being a prominent feature. (Fig. 7).

Other sections showed the same general features, the primary lesion in all being in the lining of the smaller bronchioles. Monkey 767 which showed very little consolidation gave sections in which practically the only lesions were bronchiolar (Fig. 5). No lesions were seen in sections of the nasal mucosa of one monkey M792 killed three days after infection.

Immunity to Experimental Influenza in Monkeys.

It has already been mentioned that with one exception (M717) all monkeys which had received an intratracheal inoculation of influenza virus and were given a second similar inoculation failed to give a significant temperature response. This held irrespective of whether the inoculation was of the same or of different antigenic type. It has also been noted that despite the

absence of pyrexia each of these second inoculations induced a distinct antibody rise, indicating that infection did actually occur. When the more severe infections by "W.S. egg" strains were being studied an experiment was planned to provide a preliminary indication of the effectiveness of three types of immunization against the experimental disease.

The strain "W.S.E." (monkey passage) was used throughout for immunizing and test inoculations. The immunizing material was a pool of fluids from the embryos infected amniotically with the filtrate from M767. Portion of this was formalinized by the addition of formalin to give a concentration of 0·2 p.c. formaldehyde and stored in the refrigerator. Tested 24 hours later on the chorioallantois no virus activity was manifested.

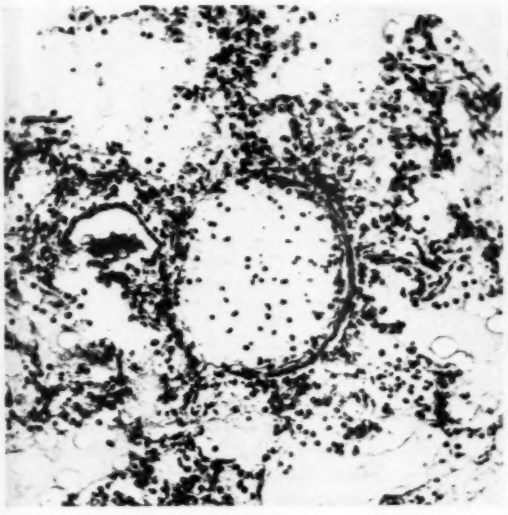

Fig. 6. Fully developed lesion in Monkey 755 dying four days after inoculation. Bronchiole in the centre shows complete loss of epithelium and like the surrounding alveoli is filled with coagulum containing relatively few cells. Interstitial pneumonia. × 160.

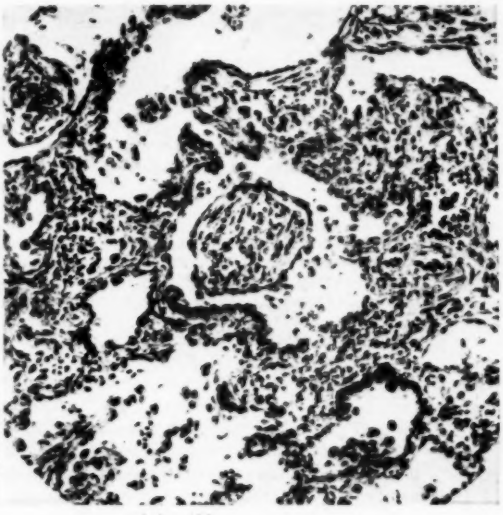

Fig. 7. To show stage of repair in Monkey 797 killed ten days after inoculation. × 160.

Two monkeys, M770 and M772, were given living virus (about 0·5 c.c.) intranasally on a single occasion three weeks before test. Neither showed a temperature rise.

Two, M778 and M793, were given two inoculations of formalinized virus (0·5 c.c.) subcutaneously three and two weeks before test.

Two, M794 and M795, received similar inoculations of untreated living virus. M794 died from unrelated causes before being tested.

Two survivors from the intratracheal passage series were included, M768 and M777 (see Table 2). These had been inoculated four and three weeks respectively before being tested.

Two normal monkeys also received the test inoculation (M796 and 797). The virus used for test was from eggs inoculated with the virus that had been used to infect M755 and M757. Its titre on the chorioallantois was $2{\cdot}5 \times 10^6$ per 0·05 c.c. and 1·0–1·5 c.c. was administered, depending on the size of the monkey. All the immunized monkeys were bled before the test inoculation and the antibody titre against "W.S.E." determined. Temperatures were recorded twice daily and four days after infection all the animals were radiographed. Only in one animal were the radiological findings equivocal so this monkey M772 was killed and examined immediately afterwards. Table 3 summarizes the findings in this experiment.

With the necessary reservations on account of the small number of animals, the indications from this experiment are (1) After an infection induced by tracheal inoculation there is an effective resistance to symptomatic re-infection. (2) A similarly effective resistance is produced by two subcutaneous injections of living virus. (3) Formalinized virus given subcutaneously is much less effective. (4) There is little increase in resistance after intranasal

inoculation of living virulent virus. (5) There is an excellent correlation between the level of circulating antibody and the degree of resistance to infection.

TABLE 3.

Immunization against experimental influenza in monkeys.

Monkey.	Mode of immunization.	Antibody titre before test.	Maximum rise above normal temperature.	X ray at 4 days.	Final result.
770	Living virus I.N.	0·6%	2·6° F.	++	K 10d. Almost complete resolution
772	„ „ „	0·2%	nil	±	K 4d. + lesions
778	Form. virus s.c.	0·5%	0·4° F.	—	K 7d. no lesions
793	„ „ „	13%	2·6° F.	++	K 7d. Healing lesions
795	Living virus s.c.	0·065%	nil	—	
768	Living virus i.tr.	0·023%	nil	—	
777	„ „ „	0·025%	nil	—	
796	Normal	N.T.	3·3° F.	—	
797	„	N.T.	3·1° F.	++	K 10d. Healing lesions

Antibody titre is expressed as the percentage to which the count of foci of "W.S. egg" is reduced by the addition of undiluted serum: the smaller the percentage the more potent the serum.

It will be noticed that all these indications are in accord with work on the immunity of ferrets and mice to influenza virus infections with the exception of the fourth. A small dose of active virus given intranasally to a ferret without anaesthesia produces the typical illness and full immunity; given similarly to mice little or no consolidation is produced but there is a high grade subsequent immunity. The possibility that in this respect human conditions resemble those in the monkey rather than in the mouse or ferret will be considered later in the discussion. Despite the extensive lesions shown radiologically in monkeys 770, 793 and 797 the animals did not become seriously ill and all had definitely recovered when killed seven or ten days after inoculation.

DISCUSSION.

In working with large and relatively expensive animals which particularly at the present time can only be obtained with difficulty it seemed better to attempt a superficial survey of several aspects of influenza virus infection in cynomolgus monkeys rather than to attempt an adequate investigation of any one aspect. There are several points of interest which arise from the present series of investigations in addition to the demonstration that well marked and sometimes fatal influenza virus infections can be produced in a species not hitherto known to be susceptible.

It is perhaps only an interesting coincidence that the intensity of the infection produced as judged by symptoms and antibody response becomes progressively less with strains isolated in 1933, 1935, 1939 and 1940. This parallels the experience of workers in the Northern Hemisphere that 1937 strains were not in general so virulent or readily adapted as "W.S." and that in subsequent years influenza A strains have become weaker in the sense that they are more difficult to isolate and adapt to other species of host. Any interpretation of these apparent large scale changes in virulence will probably be impossible until research on influenza viruses has been carried on over a period sufficiently long to include a major pandemic. In connection with the susceptibility of the lower primates to influenza it is of interest that it was reported in the English press during October, 1918, that baboons in South Africa were dying in large numbers reputedly from influenza.

The sometimes fatal infections induced by intratracheal inoculation of high titre "W.S.E." virus can be ascribed with certainty to the action of the virus and not to some accidentally associated pathogen. The strongest evidence for this is the effectiveness of various immunization procedures in preventing evidence of infection being manifested plus the failure to isolate any other type of virus or bacteria. All the evidence we have obtained is compatible with the view that the pathological and immunological changes observed were primarily due to infection of respiratory epithelium by the influenza virus strains administered and this view will be adopted throughout the discussion.

In order to produce symptoms, lung lesions or any considerable immunological response it is necessary to administer the virus directly into the trachea; simple intranasal inoculation gives no symptoms and only a weak antibody response. This is more analogous to the behaviour of the virus in mice than it is to ferret infections. Lung lesions in mice are in general produced only when the virus is introduced actually into the bronchial system by inoculation under anaesthesia. Intranasal infection of unanaesthetized mice produces as a rule immunity without more than trivial lung lesions. In the monkey the histological examinations suggest that the tracheobronchial epithelium is relatively resistant to virus infection but the bronchiolar epithelium much more susceptible. Symptomatic infection then only becomes possible when the inoculation is done in such a way that some of the inoculum reaches the smaller bronchi or bronchioles; under normal conditions there is no natural spread from the upper respiratory tract down to the bronchioles.

The next point of interest in the monkey disease is the regularity with which each successive tracheal inoculation produces a further increase in antibody. The dose given was one which if injected subcutaneously would have produced a considerable rise in antibody and it is perhaps not quite safe to eliminate the possibility that the subsequent responses were due to absorption of virus antigen from the inocula without multiplication. It is much more probable however, that the second and subsequent antibody rises are due to multiplication of virus in the respiratory epithelium. If so we must regard the immunity produced as being insufficient, even when only a few weeks have elapsed since infection, to prevent infection of cells exposed to direct contact with the virus. In the great majority of cases however a previous intratracheal infection is sufficient to prevent a subsequent inoculation of any type of influenza virus from inducing temperature rise or lung lesions visible by X-rays. The apparent discrepancy between these two findings probably depends on the fact that by intratracheal inoculation infective material can be brought into direct contact with susceptible cells. In the immune animal there is no antibody available to prevent primary infection but with the initiation of bronchiolar epithelium infection the early inflammatory response is presumably sufficient to minimize secondary virus infection of primarily uninfected epithelial cells.

Insufficient monkeys were available for an adequate test of immunizing procedures but the experiment shown in Table 3 was technically satisfactory and offers good presumptive evidence that immunity to influenza in the monkey is determined by the same factors which have been found to operate in other species. A high level of antibody may be produced either by intratracheal infection or by two subcutaneous inoculations of active virus and both methods are effectively immunizing. Formalinized virus given subcutaneously is less effective, giving very little antibody and no protection in one monkey but effective protection in the other. Living virus intranasally has never produced symptoms and the antibody response was small. Little or no protection against intratracheal infection was afforded. The failure of nasal immunization is to be ascribed to the fact that the nasal epithelium like that of the bronchi is relatively very insusceptible to infection and insufficient virus multiplication occurs to provide an adequate antigenic stimulus.

The experiments described are consistent with the generally held view of the pathology of influenza in man and experimental animals, viz., that the infection is limited to the respiratory epithelium. They suggest however that perhaps more stress should be laid on the particularly high susceptibility of the bronchiolar epithelium and relative resistance of the lining of the trachea and main bronchi.

On the immunological side the experiments indicate the importance of antibody level in determining resistance to symptomatic infection but at the same time provide evidence that even a very high antibody level is unable to prevent infection completely.

SUMMARY.

Cynomolgus monkeys *(Macaca irus)* are susceptible to infection with influenza virus strains when high titre material is administered intratracheally.

A series of fatal infections were produced with an egg-adapted strain of "W.S." in the absence of secondary bacterial infection.

The infection involves predominantly the epithelium of the smaller bronchioles, the upper respiratory epithelium (nasal cavity and bronchi) showing no histological evidence of damage.

The amount of virus recoverable from washings of the trachea and bronchi is highest at two days (the earliest period tested) and falls thereafter irrespective of whether the infection is fatal or not.

There is an active serological response to infection, several features of which are discussed in the text.

Re-infections give as a rule no temperature response but produce a sharp increase in antibody.

Two monkeys given repeated intratracheal inoculations of human strains developed high titre antibody against "Swine" influenza virus.

Limited experiments on immunization indicated (a) recovery from a preceding intratracheal infection with active virus gave a solid immunity against symptomatic re-infection, (b) subcutaneous inoculation of living virus gave effective immunity, (c) subcutaneous inoculation of formalinized virus was not effective, (d) intranasal instillation of active virus was also ineffective, (e) the degree of immunity was correlated with the antibody level.

Acknowledgment. I am indebted to Miss M. Foley for assistance in many of the antibody titrations recorded in this paper.

REFERENCES.

Burnet, F. M. (1936): Med. Res. Council, Sp. Rep. Series No. 220.
Burnet, F. M., Keogh, E. V. and Lush, D. (1937): Austral. J. exp. Biol., 15, p. 227.
Burnet, F. M. and Lush, D. (1940): *Ibid.*, 18, p. 49.
Burnet, F. M. and Foley, M. (1941): Med. J. Austral., 1, p. 68.
Long, P. H., Bliss, E. A. and Carpenter, H. M. (1931): J. Amer. Med. Assoc., 97, p. 1122.

GROWTH OF INFLUENZA VIRUS IN THE ALLANTOIC CAVITY OF THE CHICK EMBRYO[1]

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(Accepted for publication 18th August, 1941.)

In previous papers it has been shown that the allantoic fluid from embryos inoculated amniotically with influenza virus contains large quantities of virus (Burnet, 1940). This was tentatively considered as being ultimately derived from the respiratory tract of the embryo, the virus being swallowed and passing through the allantoic stalk to the allantoic cavity. A stimulus to reconsider this interpretation came from the paper by Nigg *et al.* (1940). They found that a high yield of complement-fixing antigen could be obtained from the membranes of chick embryos which had been inoculated into the allantoic cavity by injecting through the chorioallantois. On using a similar technique we found that after two or three days the allantoic fluid had a virus content of the same order as is obtained after amniotic inoculation. The technique of such inoculations can be made extremely simple and it seemed likely that a method of this sort might become of value for the rapid production of high titre virus should any effective method of immunization be developed in the future.

TECHNIQUE.

The first experiments were made following the usual amniotic technique as far as the dropping of the chorioallantois. The chorioallantois was then picked up with forceps and the inoculum placed in the allantoic cavity with a sharp pointed capillary pipette.

Practically all the experiments to be reported were made by a much less elaborate technique. Twelve or ten day eggs were candled and a point marked where the chorioallantois was well developed but where there were no large blood vessels immediately subjacent. A small cut through the compact layer of the shell was made at this point, the shell membrane being left undamaged. The inoculation was made by syringe with a fine bore needle. The point of the needle was introduced about 3 mm. into the egg and 0·05 c.c. inoculated. Subsequent incubation was at 35° C. for the required period of time.

Since we were primarily interested in the applicability of the method for the growth of virus strains not previously adapted to embryonic tissues most of the experiments have been made with "Melbourne" mouse strain. The intranasal inoculation of mice is a simpler method of titration than amniotic inoculation of chick embryos and was therefore used in all experiments where it was practicable. Several other strains and substrains were used in other experiments.

The Rise and Fall of Virus in the Allantoic Fluid after Inoculation.

Two sets of experiments with "Melbourne" mouse strain are shown in Fig. 1. In one, eggs at the twelfth day of incubation were used, in the other ten-day eggs. Titrations were made in mice, using hundred fold dilutions with three mice inoculated from each dilution. The values shown are interpolated from the results to show the dilution at which the fluid would produce average grade 2 lesions in mice. This level is approximately 100 times the dilution killing mice within seven days and 1/100 of the dilution producing minimal lesions in 50 p.c. of mice. The maximal yield is obtained at the second day after inoculation and thereafter there is a variable decline. In some eggs virus has practically disappeared in six or seven days. In others a fairly high titre persists as long as there is any allantoic fluid remaining—it is completely absorbed at the time of hatching. The changes in titre of "Swine" mouse virus inoculated suballantoically in twelve-day eggs were similar. At two days the allantoic fluid diluted 10^{-6} gave average grade 3 lesions (titre 3×10^6) while at seven days undiluted fluid produced a small lesion in only one of three mice inoculated.

The physical appearance of the allantoic fluid in the late stages of hatching varies con-

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[1] A preliminary account of this work was given in a note published in Austral. J. Science, 1941, 3, p. 133.

[2] Work carried out with the aid of grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

siderably from embryo to embryo in amount, viscosity, pigment and the form in which urate precipitates are dispersed. It is reasonable to assume that some of these physico-chemical variables will influence the titre of virus but no specific experiments have been made in the subject.

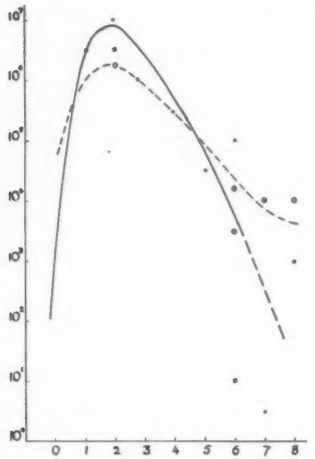

Fig. 1. Multiplication of strain "Melbourne" in allantoic cavity of 12 day (continuous line) and of 10 day (broken line) embryos. For description see text.

In adult animals the fall of virus titre about seven days after infection is generally ascribed to antibody production. It seemed worth while to make a few experiments to see whether this might function in the embryo. It is well known that antigens inoculated into the chick embryo do not produce an antibody response but no previous experimenters had used an antigen which multiplies freely in the chick tissues. Two satisfactory blood samples were obtained (1) from an unhatched chick nine days after inoculation at ten days, and (2) from a chick hatched five days previously from an egg inoculated at twelve days. The sera were separated and titrated on the chorioallantois. No trace of antibody was detectable, the count of foci when "Melbourne" egg virus diluted 1: 1,000 was mixed with saline was 28, 25, 21, 17; with chick serum 1 the count was 34, 30, 22, 15 and with chick serum 2 it was 30, 24, 24, 10.

Two incidental findings from these experiments are of interest, first that there is a very regular development of the virus in all eggs inoculated by the simple injection method and second that despite the presence within the shell of very large amounts of virus the embryo appears to be unharmed. A number of deaths occurred in eggs left to hatch but this may be in part or wholly due to the unphysiological conditions under which the later part of incubation was carried out—35° C. without turning. In the experiment shown with twelve-day eggs in Fig. 1, of eighteen eggs opened one to seven days after inoculation only two were dead. The four remaining were left to hatch but failed to do so. On examination at the twenty-third day of incubation one had died early, the other three at nineteen to twenty-one days. In another experiment shown by crosses in Fig. 1, nine twelve-day eggs were inoculated and four opened at two and six days. Three of the remaining five hatched successfully and one of the chicks was quite healthy when killed for bleeding at five days after hatching.

Growth of Unadapted Influenza Virus Strains.

Three strains of virus in the form either of ferret turbinate emulsion or chick embryo fluids which were not more than three passages removed from the original human isolation were inoculated sub-allantoically and the virus content of the allantoic fluid taken three days later was tested amniotically. The strains were "H." (Burnet and Lush, 1940) in the form of turbinates from the original ferret inoculated with throat washings, "M.N." re-isolated after experimental human infection (Burnet and Foley, 1940) and "McD." (Burnet and Foley, 1941) both in the form of embryonic fluids of third and first passage respectively.

In each case the fluids from eggs three days after inoculation with these strains were tested at hundred fold dilutions to 10^6 and all were found capable of producing specific infection in chick embryos at this dilution.

TABLE 1.

Multiplication of unadapted strains in allantoic cavity.

Strain.	Passage.	Amniotic titration. 10^{-6}	10^{-7}	10^{-8}
"H"	1	+ + (+)		
"M.N."	1	+ (+)		
	2	+ (+)	+ +	(+) — —
"McD."	1	+ + (+)		
	2	(+)	+	—

\+ Infection with typical tracheal smear.
(+) Embryo dying at three days or later, presumably from specific infection.
— Embryo surviving without lesions at four days.
Early deaths or contaminated embryos are not recorded.

A second sub-allantoic passage of the strains "M.N." and "MeD" was also made and the three-day allantoic fluids titrated to 10^8. The details of the combined titrations are shown in Table 1.

It is clear that virus unadapted to mouse or egg passage will multiply as freely by this method as will mouse-adapted virus.

Site of Virus Multiplication.

There is strikingly little to be seen macroscopically in infected eggs; the chorioallantois and the amnion appear normal. The allantoic fluid is slightly turbid and our impression is that urates do not precipitate so abundantly from infected fluids as from normal ones of the same period of incubation. Smears made from the centrifuged deposit of an infected allantoic fluid show mostly badly disintegrated cells but some cells can usually be seen with changes closely resembling those found in tracheal fluid smears from specific infections. The most characteristic of these are cells whose nucleus has broken up into a small number of deeply stained spherical bodies. Sections of the chorioallantois show only minor changes in the allantoic (entodermal) epithelium which retains its character as a single cell layer. There are however single cells or small groups of adjacent cells which show damage presumably from virus infection. These may be moderately swollen, with cytoplasmic vacuolation and poorly staining nucleus, or they may show the characteristic fragmented nucleus which is common in all types of influenza virus lesions in embryonic tissues. These cells can also be recognized in impression preparations made by the method of Burnet and Rountree (1935) but with the allantoic instead of the chorionic surface against the glass.

If fluid is removed at the optimal period for virus multiplication, 48 hours, a light turbidity of the fluid not due to urates or blood appears to be almost diagnostic of infection. Normal allantoic fluid at this stage is perfectly limpid except in unduly dehydrated eggs when a urate precipitate may be present. This will dissolve readily if two to three volumes of warm saline are added. A further check on the fact of infection is given by the appearance of the typical disintegrated nuclei in smears of the cellular deposit.

Immunizing Capacity of Fluids at Different Stages.

In view of the fact that any virus liberated into the allantoic fluid would be expected to accumulate there, it seemed possible that at the later stages of incubation a high concentration of dead virus particles and their disintegration products might be present. This might have an immunizing value out of proportion to its virus content. The following experiment was therefore made to determine the antigenic value of fluid taken at two, four and seven days after inoculation:

Eggs were inoculated sub-allantoically with "W.S." mouse passage strain and incubated at 35° for two, four and seven days. Allantoic fluids from two eggs were pooled for the second- and fourth-day fluids but only one suitable seven-day fluid was obtained. The pooled fluids were titrated for virus content in mice and used for immunization of a series of mice by subcutaneous inoculation. Two doses at seven days' interval of 0·25 c.c. were given either of undiluted or diluted fluid and the mice tested against the homologous strain "W.S." and an antigenically distinct strain "Melbourne". The test inoculum was a 1: 100 dilution of stock mouse virus and represented ten to one hundred lethal doses.

The results are shown in Table 2. It will be seen that both homologous and heterologous immunity is highest with the two-day fluid but that the seven-day fluid is considerably more effective as an immunizing agent than would be judged from its very low content of living virus.

Concomitantly with this experiment a series of mice were immunized with an emulsion prepared by grinding the membranes removed from the same two-day eggs. The whole of the

TABLE 2.

Fluid.	Titre.	Dose used for immunization.	Immunity test. "Melbourne".	"W.S."
2 day	1×10^6	0·0025 c.c.	4^5, 4^6, 1, 0	1, 0, 0, 0
		0·025 c.c.	4^5, 4^6, 0, 0	1, 0, 0, 0
		0·25 c.c.	4^5, 1, 0, 0	0, 0, 0, 0
4 day	3×10^5	0·0025 c.c.	4^6, 4^7, 4^7, 3	3, 3, 2, 2
		0·25 c.c.	2, 2, 1, 1	0, 0, 0, 0
7 day	3×10^2	0·025 c.c.	4^7, 4^7, 4^7, 2	3, 3, 2, 0
		0·25 c.c.	4^6, 3, 2, 1	2, 1, 1, 0

Titre shown is the dilution producing average grade 2 lesions.

Immunization: two doses subcutaneously in a volume of 0·25 c.c. at seven days interval with test inoculation fourteen days after the second dose.

All controls to test inoculation died: "Melbourne" at 5, 5, 5, 5, 6, 6 days, "W.S." at 4, 5, 5, 6, 6, 6 days.

chorioallantois and most of the amnion were removed aseptically and allowed to drain away excess fluid for an hour. They were then ground with quartz powder and 1 c.c. of broth added for each embryo used. The thick suspension was allowed to settle overnight in the refrigerator and the supernatant fluid used as immunizing agent diluted 1: 10 and 1: 100. Table 3 shows the results of subsequent immunity tests. The tissue emulsion is almost exactly of 1/10 the immunizing power of fluid from the same eggs.

TABLE 3.

Activity of membrane emulsion as immunizing agent.

Fluid.	Titre.	Dose used for immunization.	Immunity test. "Melbourne".	"W.S."
2 day membrane emulsion	3×10^5	0·0025 c.c.	44, 44, 45, 45	46, 46, 3
		0·025 c.c.	45, 47, 2, 2	2, 1, 0, 0

Multiplication of Influenza Virus B by Sub-Allantoic Inoculation.

The new antigenic type of influenza virus isolated by Francis (1940) (influenza virus B) will multiply after sub-allantoic inoculation as well as by the amniotic method. The mouse-adapted B strain "Lee" was received through the kindness of Dr. Francis. After three amniotic passages during which typical lesions were produced, mixed amniotic and allantoic fluid was inoculated by the standard sub-allantoic method into four twelve-day embryos. After incubation at 35° C. two eggs were opened at two days and the other two at three days. All embryos were alive and apparently normal: the allantoic fluids were slightly turbid and smears made from the centrifuged deposit showed the damaged cells which we regard as evidence of specific infection. The allantoic fluids from the two eggs opened at 48 hours were pooled and titrated by mouse inoculation and the same procedure was followed with the three-day embryos. Table 4 gives the results.

TABLE 4.

Mouse titration of allantoic fluids from embryos inoculated with influenza virus B.

	Undiluted.	10^{-2}	10^{-4}	10^{-6}
2 day pooled allantoic fluids	43, 44, 45	44, 45, 43	3, 2, 2	0, 0, 0
3 day " " "	45, 47, 3	3, 3, 0	2, 1, 1	0, 0,

The two-day fluid was used to continue the amniotic passage series and on several occasions appropriate serological tests showed that the series had maintained the typical B antigenic characters.

As with influenza A infections the maximal titre after sub-allantoic inoculation seems to be reached at about 48 hours.

Allantoic Fluid as Antigen for Complement Fixation Reactions.

The use of embryonic fluids as antigen for complement fixation tests will be described in a subsequent paper by Miss Foley. Here we wish only to report that the allantoic fluid from eggs inoculated two days previously with the strain "W.S." (mouse) provides a very satisfactory antigen. This confirms similar findings recently reported by Henle and Chambers (1941).

DISCUSSION.

The chief theoretical interest of these results is the demonstration of the susceptibility of the entodermal allantoic cells to infection by any type of influenza virus. The ease with which unadapted strains multiply in this situation contrasts with the difficulty of obtaining chorioallantoic multiplication with such strains where the ectodermal chorionic epithelium is exposed to infection. There are however considerable physical differences between the allantoic cells bathed in fluid and the chorionic cells which under the conditions of inoculation are exposed to the air and rapidly develop a certain degree of cornification. The allantois is, like the respiratory tract, an outgrowth of the primitive alimentary canal and it is tempting to regard the susceptibility to influenza virus infection of the two tissues as being related to their common embryonic origin.

On the practical side the method provides much the simplest available method of obtaining high titre sterile virus from any desired strain of influenza virus. While this work was in progress a paper by Henle and Chambers (1941) appeared describing a similar method and recording the high virus content of the extra-embryonic fluids. They showed also that these fluids contained a high concentration of complement-fixing antigen and in view of the low protein content of the allantoic fluid they suggested its possible use as a vaccine.

If pandemic influenza should ever recur and large scale immunization be demanded, the technical methods for production of vaccine would need to be such that they could be carried through by persons with only unspecialized bacteriological training and the material required would have to be readily available. Irrespective of the form of vaccine which may eventually be developed—living or killed virus, normal or attenuated strain, administered subcutaneously or intranasally—the simple method described above seems to be the only one which might conceivably cope with a concentrated demand for hundreds of thousands of doses within a brief period of time.

It is well known that embryonic animals do not produce antibody and a number of failures to induce antibody formation by inoculation of various antigens into chick embryos has been reported (Grasset, 1929). In no previous work however has an active non-lethal infection of the embryo been produced by an agent which is a highly active antigen in adult animals. The egg contains at one stage about 10 c.c. of fluid, 0·01 c.c. of which is sufficient to immunize a mouse effectively, yet the chick nine and fourteen days after infection shows no trace of antibody by a sensitive technique. This provides particularly strong evidence of the absence of any antibody producing mechanism during embryonic life.

SUMMARY.

Free multiplication of any type of influenza virus (A or B) follows inoculation into the allantoic cavity of the developing chick embryo at ten to twelve days incubation.

The allantoic fluid taken two to three days after infection contains large amounts of virus and complement-fixing antigen.

Mice can be immunized effectively against the homologous strain with two subcutaneous doses of 0·0025 c.c. of infected allantoic fluid.

Virus multiplication takes place in the entodermal cells lining the allantoic cavity.

With some strains the embryo remains unaffected and a proportion of chicks hatch normally: no antibody against influenza virus is developed.

REFERENCES.

Burnet, F. M. (1940a): Brit. J. exp. Path., 21, p. 147.
Burnet, F. M. (1940b): Austral. J. exp. Biol., 18, p. 353.
Burnet, F. M. and Foley, M. (1940): Med. J. Austral., 2, p. 655.
Burnet, F. M. and Foley, M. (1941): *Ibid.*, 1, p. 68.
Burnet, F. M. and Lush, D. (1940): Austral. J. exp. Biol., 18, p. 49.
Burnet, F. M. and Roumtree, P. (1935): J. Path. Bact., 40, p. 471.
Francis, T. (1940): Science, 92, p. 405.
Grasset, E. (1929): Publ. Sth. Afr. Inst. Med. Res., 4, p. 171.
Henle, W. and Chambers, L. A. (1941): Proc. Soc. exp. Biol. and Med., 46, p. 713.
Nigg, C., Crowley, J. H. and Wilson, D. E. (1940): Science, 91, p. 603.

CIRCULATORY AND OTHER EFFECTS OF THE TOXIN OF *CL. SEPTIQUE*

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Dale in a note appended to a paper by Robertson (1919) suggested on the basis of a few experiments on rabbits that in this animal the toxin of *Cl. septique* has a double action, pressor, presumably due to vasoconstriction, and toxic on the heart muscle. Lautenschläger (1920) in a more extensive study in Straub's laboratory emphasized the toxic effect upon the heart muscle which he and Straub (1919) regarded as closely resembling that of digitalis. Buttle and Trevan (1928) studied the specific contraction caused by the toxin in the rabbit's uterus and intestine and showed that the action of the toxin on the isolated auricle of the rabbit differed from that of digitalis, there being no initial increase in the extent of the heart beat before its progressive diminution and cessation.

In the present paper we record the results of studies on the circulatory effects of the toxin in the cat and rabbit, on the liberation from perfused tissues of pharmacologically active substances in injury caused by it and on its effects on some kinds of smooth muscle other than those studied by Buttle and Trevan.

METHODS.

The *Cl. septique* toxin used in our earlier experiments was obtained by the courtesy of Dr. J. W. Trevan of the Wellcome Laboratories. For later experiments we used toxin made by the Commonwealth Serum Laboratories. Both these toxins had a m.l.d. for 20 gm. mice of approximately 0·18 mg. The C.S.L. toxin contained traces of a histamine-like impurity. The antitoxin was a concentrated serum made by the Commonwealth Serum Laboratories. It neutralized more than 2 gm. of toxin per c.c. For making neutralized mixtures we measured the antitoxin with a Trevan micro-syringe.

Cats and rabbits were anaesthetized with chloralose, 10 c.c. of 1 p.c. solution per kg. intravenously. For operations some ether was used in addition. The vagi were cut, a tracheal cannula inserted and artificial ventilation given. The arterial blood pressure was recorded from a carotid artery, the venous pressure from the right external jugular or the right innominate vein, the cannula in the vein being connected through a water manometer to a Brodie tambour. The pressure in the pulmonary artery was recorded by the method of Kellaway and LeMessurier (1936). Heparin (50 units), or chlorazol fast pink (1 c.c. of 8 p.c.) was used in the recording cannula in vein or pulmonary artery. We studied the effect of toxin on the vessels of the hind limb by the method of Feldberg and Schilf (1930), the blood being made incoagulable by the injection of 1 c.c. per kg. of 8 p.c. chlorazol fast pink. The toxin was injected into the cut stump of the lateral circumflex artery and the outflow of blood from the femoral vein was recorded by counting drops, the blood being returned continuously into the left jugular vein. Loss of fluid from the circulation was estimated by determining the haemoglobin concentration by the Sahli method, the blood samples being collected with a syringe by arterial puncture.

Perfusion Experiments.

The lungs of guinea-pigs were perfused as in the experiments of Feldberg and Kellaway (1937), the rate varying from 1 to 2 c.c. per minute. The perfusate was tested for histamine and slow-reacting muscle-stimulating substances on the isolated jejunum of the guinea-pig suspended in oxygenated Tyrode solution in a bath of 7 c.c. The histamine estimated is expressed as dichloride. Titrations of histamine were checked by estimation on the blood pressure of atropinized eviscerate cats. The liver of rabbits was perfused through the portal vein as described for the liver of the dog (Feldberg and Kellaway, 1938). Adenyl compounds in the perfusate were estimated on the heart of the atropinized guinea-pig by the method of Drury, Lutwak-Mann and Solandt (1938), the cardio-depressant effects being matched with adenosine (B.D.H.). The perfusate was collected, unless otherwise stated, over a boiling water bath. Extracts of organs

[1] This work was aided by a Grant from the National Health and Medical Research Council,

for estimation of histamine and adenyl compounds were made by grinding weighed amounts with finely divided silica in Tyrode solution (1: 50 or 1: 20) and boiling. Perfusion of the hearts of cats and rabbits was carried out as described by Feldberg and Kellaway (1938) using Gunn's modification (1913) of Langendorff's method.

Experiments on Plain Muscle.

Pieces of jejunum of guinea-pig were suspended in oxygenated Tyrode solution and single horns of the uteri of virgin guinea-pigs and rats in oxygenated anaphylactic Ringer's solution in a 7 c.c. bath.

RESULTS.

Circulatory Effects in the Cat.

(i) *Effects upon the blood pressure in the carotid artery and upon the venous pressure.* The toxin of *Cl. septique* injected intravenously in doses of from 25-40 mg. per kg. into cats under chloralose anaesthesia causes death in from 4 to 60 minutes. Within a few seconds after the injection there is a sharp fall of pressure in the carotid artery resembling the response to a small dose of histamine. The pressure rapidly recovers, sometimes showing a sharp transient after-rise with acceleration of the heart beat. Thereafter the systemic blood pressure falls gradually or more quickly to zero. In a few experiments a secondary rise of systemic pressure commencing about 5 minutes after the injection preceded the final fall of blood pressure. At death there are extensive haemorrhages in the lungs or haemorrhagic oedema; the right auricle is distended and the left heart relatively empty. When neutralized toxin is injected, apart from early circulatory disturbances, the blood pressure in the carotid artery is well maintained and no morbid changes are found in the lungs when the animals are killed.

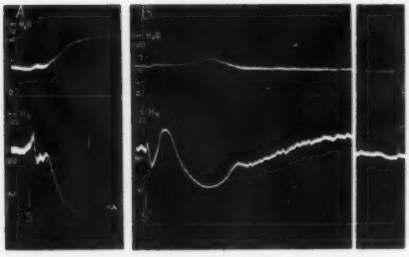

Fig. 1. Records of the blood pressure in a carotid artery (lower tracing) and in a jugular vein (upper tracing) from two cats. At S, 100 mg. of *Cl. septique* toxin and at S′, 100 mg. of neutralized toxin were injected intravenously. The interval between the panels of B was 10 minutes. Time in half minutes. Details in text.

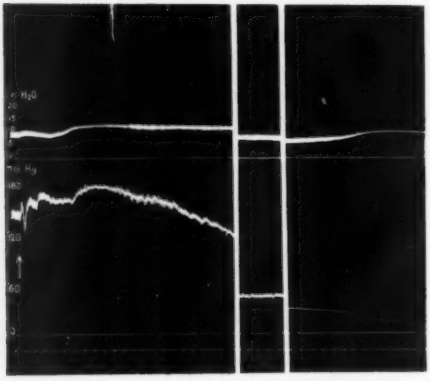

Fig. 2. Records of the blood pressure in a carotid artery (lower tracing) and in an innominate vein of a cat weighing 2·4 kg. At the arrow 60 mg. of *Cl. septique* toxin were injected. The intervals between the panels were 19 and 26 minutes. Details in text.

Fig. 1A from a cat weighing 3·5 kg. which died in 4½ minutes after the injection of 100 mg. of toxin shows an unusual rise in systemic blood pressure immediately after the injection quickly followed by a histamine-like depressor effect. Within a minute there was a steep terminal fall of systemic blood pressure, the venous pressure at the same time rising steeply and being maintained until death. Fig. 1B shows the effect of the intravenous injection of 100 mg. of neutralized

toxin in a cat weighing 1·8 kg. The initial histamine-like fall and recovery are obviously non-specific effects. The following fall of systemic blood pressure from which also recovery took place was attended by a transitory rise in the venous pressure. Part at least of the early circulatory effects of the toxin are therefore non-specific. In this animal after recovery from these early effects the systemic blood pressure was well maintained and the venous pressure returned to its normal level and remained there. After 45 minutes the animal was killed and the lungs were found to be normal.

In several experiments heart block was observed during the early fall of systemic blood pressure. In experiments later to be described in which the thorax was open and the pressure in the pulmonary artery was recorded, heart block was also observed as a terminal event.

In experiments in which the injection of toxin caused death with a gradual terminal fall of systemic pressure in from 50 to 60 minutes the terminal venous pressure was not greatly increased. The lungs usually showed striking haemorrhagic oedema. In Fig. 2 from a cat weighing 2·4 kg. in which 60 mg. of toxin were injected intravenously the venous pressure began to rise about 2 minutes after the injection; it was maintained for about 20 minutes and then gradually fell, rising once more when the systemic blood pressure fell terminally an hour after the injection.

After smaller doses of toxin (6 to 20 mg. per kg.) death occurs with oedema of the lungs usually in from 1 to 2½ hours though two animals which received 12 and 20 mg. per kg. died with severe pulmonary oedema in 22 and 27 minutes. An immediate transitory depressor effect was observed in some experiments. This was non-specific. The gradual fall of systemic blood pressure during these prolonged experiments was sometimes preceded by a short period of raised blood pressure commencing 3 to 5 minutes after the injection.

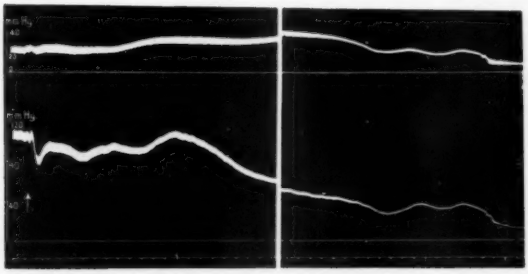

Fig. 3. Records of the blood pressure in a carotid (lower tracing) and in a pulmonary artery (upper tracing) from a cat weighing 2·0 kg. At the arrow 60 mg. of *Cl. septique* toxin were injected intravenously. The interval between the panels was 5 minutes. Time in half minutes. Details in text.

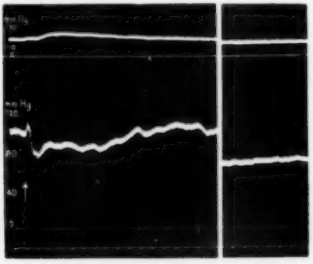

Fig. 4. Records of the blood pressure in a carotid (lower tracing) and in a pulmonary artery (upper tracing) from a cat weighing 2·2 kg. At the arrow 60 mg. of neutralized toxin of *Cl. septique* were injected intravenously. The interval between the panels was 30 minutes. Time in half minutes. Details in text.

(ii) *Effects upon the blood pressure in the pulmonary artery.* In cats under chloralose anaesthesia with the thorax open and the pressure in a pulmonary artery continuously recorded, the intravenous injection of from 30 to 50 mg. of toxin per kg. causes death in from 7 to 26 minutes. The changes in the systemic blood pressure are similar to those already described. The initial histamine-like fall of systemic pressure is generally attended by a small rise in the pressure in the pulmonary artery. After half to three minutes a secondary rise takes place in the pressure in the pulmonary artery, followed by a rapid or gradual fall in the systemic blood pressure. The pressure in the pulmonary artery is maintained at a high level until the heart finally fails. The amplitude of pulsations in the pulmonary manometer is well maintained whereas that of pulsations in the systemic manometer is progressively diminished.

In these experiments it was possible to observe, a few minutes after the injection of toxin, the gradual emptying of the left auricle of blood and the simultaneous development of oedema of the right lung. Fig. 3 from a cat weighing 2 kg. in which 60 mg. of toxin were injected intravenously illustrates most of these changes. The effect on the pressure in the pulmonary artery accompanying the immediate histamine-like depression of the carotid pressure was insignificant. The pressure in the pulmonary artery began to rise 3 minutes after the injection and 3 minutes later there was a progressive fall in the systemic blood pressure, the pressure in the pulmonary artery continuing to rise.

The injection of neutralized toxin in these doses causes only the initial circulatory disturbances—an almost immediate histamine-like depression in the systemic blood pressure and a trivial and transitory rise in the pressure in the pulmonary artery. Fig. 4 shows the effect of a dose of 60 mg. of neutralized toxin in a cat weighing 2·2 kg. There was a small rise in the pressure in the pulmonary artery 45 seconds after the injection. This did not correspond to the initial histamine-like depression in the systemic blood pressure but was related to a more prolonged depression of the systemic pressure lasting for 3 minutes. This early depression of systemic pressure following the histamine-like effect was observed in varying degrees in several experiments and was clearly non-specific. After neutralized toxin there was no oedema of the lung and the left auricle was normally filled with blood.

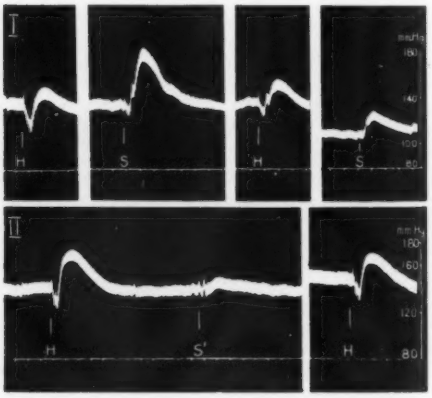

Fig. 5. Records of the carotid blood pressure of two eviscerated cats. In I at H 1γ histamine and at S 10 mg. of *Cl septique* toxin in 0·5 c.c. saline were injected into the cut stump of the coeliac artery. The intervals between the panels from left to right were 12, 15 and 19 minutes. In II at H 1γ histamine and at S′ 10 mg. of overneutralized toxin were injected into the coeliac artery of a second preparation. The interval between the panels was 8 minutes. Time in half minutes.

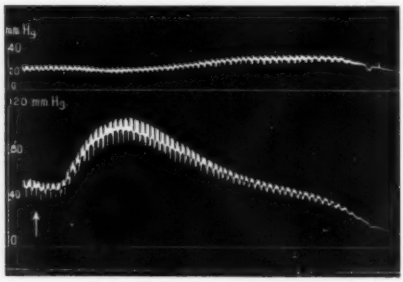

Fig. 6. Records of the pressure in a pulmonary artery (upper tracing) and in a carotid artery (lower tracing) from a rabbit weighing 2·5 kg. At arrow 5 mg. *Cl. septique* toxin were injected. Time in half minutes.

(iii) *The liberation of adrenalin from the suprarenal glands.* The toxin injected intravenously in doses of 5 to 10 mg. is without effect on the carotid blood pressure. Doses of this size when injected into the cut stump of the coeliac artery in eviscerated cats cause, after a latent period of 20 seconds, a sharp rise of the carotid blood pressure with quickening of the pulse rate, indicating an output of adrenalin from the suprarenal glands. This effect is specific, because neutralized toxin similarly injected in other preparations causes no significant changes in the blood pressure. It is not possible to compare the effect of different doses of toxin nor of neutralized with unneutralized toxin upon the same preparation because the responses to second doses of toxin are always diminished.

These facts are illustrated in Fig. 5. The response to a dose of 10 mg. of toxin injected intra-arterially is shown at S in Fig. 5 I. It differs from that to 1γ of histamine (at H) in the small size of the initial depressor effect and in the latency of the rise of pressure (20 seconds instead of 14 seconds after histamine). The response to a second dose of toxin 41 minutes later was much smaller. In Fig. 5 II at S′ is shown the insignificant response to 10 mg. of neutralized toxin.

(iv) *Effects on the vessels of the hind limb.* In 7 experiments in which the toxin in doses ranging from 1 to 10 mg. was injected into the cut stump of the lateral circumflex branch of the femoral artery no regularly significant changes were observed in the outflow from the common femoral vein.

In a cat weighing 4·2 kg. under chloralose anaesthesia the intra-arterial injection of 1γ histamine caused striking immediate vaso-dilatation in the limb. The outflow measured in drops before the injection was 30 per 10 seconds; in the first 10 seconds after the injection it rose to 44 drops; during the succeeding 20 seconds it poured out continuously and returned to normal within one minute. Apart from a trivial immediate volume effect the injection of 5 mg. of toxin of *Cl. septique* caused no significant change in outflow during 11 minutes after which 1γ histamine gave a dilator response identical with that observed before the injection of toxin. Adrenalin chloride 5γ gave a transitory constriction of the limb vessels, the outflow before being 28 drops per minute and in successive 10 second intervals after the injection 26, 14, 21, 26, 28, 32 and 29.

(v) *Effects on the coronary circulation.* The toxin in doses of 5 to 20 mg. causes dilatation of the coronary vessels lasting from 15 to 30 minutes. In larger doses (40 mg.) it causes a strong constriction from which no recovery takes place; the heart in one experiment ceased to beat in 15 minutes. Neither of these effects is wholly specific, as can be seen from the results of the six experiments summarized in Table 1.

TABLE 1.

Effect of the toxin of Cl. septique *on the isolated perfused heart of the cat.*

Dose.	Before injection	Coronary flow in c.c. per minute every 2nd minute after injection.
5 mg.	13	13, 15, 16·5, 16, 15, 14, 15, 14, 14·5, 14, 13·5, 13·5, 12·5
5 mg. (neutralized)	18	17·5, 17, 16·5, 16, 15, 14, 14, 14, 13, 13, 12
20 mg.	13	23, 29, 28, 29, 27, 24, 23, 20, 19, 17, 16, 16, 16, 15, 14, 14, 13
20 mg. (neutralized)	25	25, 33, 35, 34, 31, 30, 28, 26, 23, 22, 20, 19, 18
40 mg.	16	9, 8, 6, 5, 5, 3, 3, 2, 2,
40 mg. (neutralized)	16	12, 14, 13, 12, 11, 10, 10, 9, 9

The dilator effect of 20 mg. is much greater than that caused by 5 mg. and the dilator effect of fully neutralized toxin in these doses is less than that of unneutralized toxin. The slight constriction observed after 5 mg. of neutralized toxin is probably not significant. The constrictor effect of 40 mg. was mainly specific though neutralized toxin in this dose had a small prolonged constrictor effect.

(vi) *Loss of fluid from circulation.* In poisoning by the toxin of *Cl. septique* there is sometimes a loss of fluid from the circulation but this can be accounted for by the development of oedema of the lung and of effusion into the pleural cavities.

In 4 experiments in cats weighing 2·7, 2·8, 3·4 and 3·5 kg. in which a dose of 20 mg. of toxin was injected intravenously death resulted in 2 hours 19 minutes, 2 hours 30 minutes, 2 hours 15 minutes and 1 hour 11 minutes respectively. In the first two there was no change in the haemoglobin percentage after the injection, in the third the haemoglobin rose from 73 p.c. to 101 p.c. and in the fourth from 79 p.c. to 96 p.c. just before death. The losses of fluid from the circulation in these experiments calculated from the data of Dale and Laidlaw (1919) (blood volume of the cat 50 c.c. per kg. of which 33·3 p.c. is red blood corpuscles) were 0, 0, 47 c.c. and 31 c.c. The actual losses of fluid observed either as pleural effusion or as oedema fluid collected at death by drainage of the excised lungs were 15 c.c., 0, 42 c.c. and 36 c.c. Since failure of increase in haemoglobin concentration in some experiments is associated with absence or only slight degree of oedema of the lungs and since the increase observed in others can be accounted for almost wholly by fluid lost in the lungs and pleural spaces it appears that the toxin causes no significant loss of fluid in the periphery of the greater circulation.

Circulatory Effects in the Rabbit.

(i) *Effects upon the blood pressure in the carotid artery and upon the venous pressure.* The toxin of *Cl. septique* injected intravenously in doses of from 1 to 4 mg. per kg. causes within a minute a gradual or steep rise in the systemic blood pressure. During the second minute after a dose of 2 to 4 mg. per kg. this rise is often interrupted or terminated by cardiac failure, manifested by a sharp fall of systemic pressure and a corresponding rise of venous pressure. Recovery when it occurs is by a steep rise of systemic pressure usually with a quickened heart beat. The blood pressure then falls gradually, death occurring in from 8 to 25 minutes after the injection. This terminal fall of blood pressure is attended by a gradual rise in the venous pressure.

After a dose of 1 mg. per kg. the initial rise of blood pressure lasts for from 3 to 6 minutes and then a gradual fall occurs, death taking place in from 15 to 30 minutes. At death the right auricle is distended with blood. When death takes place rapidly (in 3 to 7 minutes) the left auricle is normally filled with blood but when it occurs only after a longer interval (15 to 30 minutes) the left auricle is empty. Even in this last group of experiments oedema of the lung is found only occasionally and is then of slight degree. All these changes are specific, neutralized toxin in these doses injected intravenously in rabbits being wholly without effect.

(ii) *Effects upon the pressure in the pulmonary artery.* The intravenous injection of the toxin in doses of 1 to 2 mg. per kg. causes only slight changes in the pressure in the pulmonary artery during the primary rise in systemic blood pressure. When this rise is interrupted by a temporary fall in systemic pressure the pressure in the pulmonary artery falls simultaneously rising again with the pressure in the carotid artery as recovery takes place. The pressure in the pulmonary artery now continues to rise and is maintained at a high level while the systemic blood pressure is falling.

Fig. 6 shows the effect of a dose of 5 mg. of toxin in a rabbit weighing 2·5 kg. Here the initial rise of systemic pressure was not interrupted by cardiac failure and the pressure in the pulmonary artery commenced to rise 4 minutes after the injection when the systemic pressure was beginning to fall. The effects of the toxin on the pressure in the pulmonary artery are likewise wholly specific, neutralized toxin being without effect.

(iii) *Effects on the systemic blood pressure in the decapitate preparation.* The initial rise in the systemic blood pressure also occurs in the decapitate preparation.

In two experiments after the injection of 2 mg. per kg. of toxin the blood pressure rose within two minutes from 22 to 41 mm. Hg. and from 30 to 48 mm. Hg. This rise was interrupted by a steep terminal fall, death occurring in 10 and 8 minutes respectively. The result of a third experiment is seen in Fig. 7. Here the initial rise of systemic blood pressure was trivial but a steep secondary rise corresponding to the recovery from the early transitory cardiac failure observed in intact animals, occurred from the 6th to the 9th minute after the injection, accompanied by quickening of the heart rate. The systemic blood pressure now fell gradually, death taking place half-an-hour after the injection.

(iv) *Effects on the systemic blood pressure after section of the splanchnic nerves and in the eviscerate preparation after excision of the suprarenal glands.* After bilateral section of the splanchnic nerves the initial rise of blood pressure still takes place when toxin in suitable dosage is injected intravenously. Even excision of both suprarenals in the eviscerate preparation does not prevent it. In this last type of experiment after the injection of 2 mg. of toxin per kg. we observed changes exactly comparable to those repeatedly seen in the intact rabbit.

A rabbit weighing 2·5 kg. was anaesthetized with chloralose; the stomach, intestines and spleen were removed and both suprarenal glands excised. The intravenous injection of 5 mg. of *Cl. septique* toxin caused within 30 seconds a rise of pressure in the carotid artery from 70 to 90 mm. Hg. The blood pressure fell 28 mm. Hg. 3½ minutes after the injection and rose again steeply to 114 mm. Hg. The final fall of pressure began 6 minutes after the injection, death occurring in 13 minutes.

(v) *Effects on the vessels of the hind limb.* The experiments recorded in the preceding sections suggest that the initial rise of systemic pressure after the injection of toxin is attributable to a direct constrictor action on the peripheral vessels. We attempted to demonstrate this directly. When a small dose of toxin (0·5 mg.) is injected into the cut stump of the lateral circumflex artery, the outflow from the common femoral vein being measured, there is a small constrictor effect lasting up to 10 minutes. This is specific since neutralized toxin in this dosage is without effect.

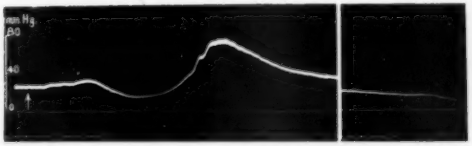

Fig. 7. Record of the carotid blood pressure from a decapitate rabbit weighing 2·4 kg. At the arrow 5 mg. of *Cl. septique* toxin were injected. Interval between the panels 9½ minutes. Time in half minutes.

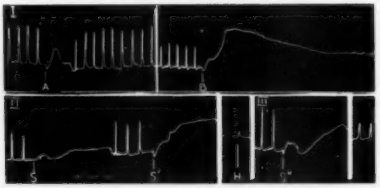

Fig. 8. Responses of 3 pieces of jejunum from two guinea-pigs to the toxin of *Cl. septique*. The response at H is to 0·5γ and the unlettered contractions to 0·1γ histamine. Time in minutes. Details in text.

In such an experiment the outflow from the femoral vein of a rabbit weighing 3 kg. was measured in drops per 20 seconds. The outflow before the injection of 0·5 mg. of toxin was 24 and in succeeding 20 seconds after 24, 25, 22, 20, 21, 20, 21, 22 persisting at this rate for 10 minutes and gradually returning to 24 drops per 20 seconds. In this preparation 1γ adrenalin chloride caused somewhat stronger constriction lasting about 7 minutes. The outflow before was 22 drops per 20 seconds and in successive intervals after was 22, 18, 16, 16, 16, 16.

The constriction was not always so prolonged as in the experiment quoted above. In another experiment the outflow for 20 seconds before the injection of 0·5 mg. of toxin was 38 drops and in successive 20 seconds after 45, 34, 31, 32, 30, 30, 34, 33, 34, 36, 36, 35, 37, 37, 38, the constriction having passed off in five minutes.

(vi) *Effects on the coronary circulation.* The effect of the toxin on the coronary circulation of the rabbit is generally similar to that in the cat except that much smaller doses are effective in causing constriction, which is mainly a specific effect.

TABLE 2.

Effects of the toxin of Cl. septique *on the isolated perfused heart of the rabbit.*

Dose.	Before injection.	Coronary flow in c.c. per minute. First and every alternate minute after injection.
0·5 mg.	10	4, 16, 15, 14, 13, 13, 13, 13, 13, 14, 14, 13, 14, 13, 13, 13
Antitoxin 0·02 c.c.	15	16, 16, 13, 12, 12, 12, 13, 13, 13
0·5 mg. neutralized	13	9, 17, 21, 17, 15, 14, 14, 14, 14
2 mg. neutralized	14	11, 11, 9, 8, 7, 7, 7, 7, 7, 7
2 mg.	7	7, 2, 1, 0·5, 0·5, 0·5, 0·5
2 mg. neutralized	9	9, 5, 4, 5, 5, 5, 6, 6, 6, 6
2 mg.	7	7, 1, 0, 0, 0, 0, 0, 0
5 mg.	15	9, 10, 10, 7, 6, 6, 4, 5, 5, 5, 5, 4, 4
5 mg. neutralized	19	8, 27, 23, 22, 22, 22, 24, 23, 22, 21, 20, 18, 17
5 mg.	17	12, 14, 8, 6, 6, 5, 5, 4, 4, 4, 4, 4, 4, 3, 2

The results of six experiments are set out in Table 2. In three of these, the 3rd, 4th and 6th in the Table, a dose of neutralized toxin and in one, the 2nd experiment, the effect of 0·02 c.c. of antitoxin alone (the neutralizing dose for 20 mg. toxin) were first tested. Rather contrary

to our expectation, the following dose of toxin appeared to exert its full effect. During the first minute after the injection there was a sharp falling off in flow which was apparently non-specific. The subsequent vasodilatation after a dose of 0·5 mg. was mainly non-specific, the effect being more prolonged than that of the same dose neutralized. Doses of 2 mg. and 5 mg. of unneutralized toxin gave strong vasoconstriction, the heart ceasing to beat in 15 to 30 minutes. The preceding dose of neutralized toxin in two experiments caused a lesser degree of constriction. In the last experiment in the Table a dose of 5 mg. neutralized gave an effect similar to that of a dose of 0·5 of unneutralized toxin.

The Liberation of Histamine from Isolated Perfused Lungs of the Guinea-pig.

When the isolated lung preparation is perfused with Tyrode solution at rates of flow from 1 to 2 c.c. per minute histamine cannot be detected in the perfusate though there is generally some in the small amount of drainage fluid collected when the lungs are cut down after perfusion for 2 hours or more. The injection of the toxin of *Cl. septique* in doses of 20 and 60 mg. into the pulmonary artery causes histamine to appear in the perfusate. This effect is not wholly specific because neutralized toxin in similar dosage also causes the liberation of histamine though in smaller amount. In none of these experiments was there any output of muscle-stimulating substances other than histamine.

In Table 3 the results of these experiments are summarized. The average total output in the perfusate in 5 experiments in which 60 mg. of toxin were injected was 13·3γ, and in 4 experiments in which the same dose of neutralized toxin was injected was 4γ. Using Student's "t" distribution we attempted to determine the significance of the difference of these means. The value obtained for "t" was 2·536 and with 7 degrees of freedom P is approximately equal to 0·04 so that the difference must be regarded as significant though not highly so. The application of Fisher's Z test showed that the variances of the two groups were not significantly different so that the difference in means contributed solely to the value of "t" obtained. The output in the perfusate was greatest from those lungs with the largest content of histamine in the tissues. There was some variation in the time of its appearance in the perfusate but generally, having appeared, it persisted throughout the remainder of the perfusion. In one only of the experiments after neutralized toxin the output was transient being confined to the first two samples. Antitoxin alone in control experiments caused no output of histamine in the perfusate.

TABLE 3.

Liberation of histamine from the perfused lungs of the guinea-pig.

Dosage in mg.	Content of histamine in lung in γ per gm.	Period during which histamine was present in the perfusate.	Total histamine liberated during perfusion in γ.	Histamine in drainage fluid in γ.	Duration of perfusion
Toxin alone					
20	35	—	—	0·3	5 hours
20	14	20 to 120 min.	6·5	Nil	2 hours
60	100	7 to 150 „	17·7	0·5	2½ hours
60	23	30 to 150 „	9·9	0·2	3½ hours
60	45	7 to 180 „	14·9	0·4	3 hours
60	42	120 to 180 „	3·7	1·3	3 hours
60	65	7 to 300 „	19·9	2	5 hours
Toxin and Antitoxin					
20	40	—	—	0·1	5 hours
60	125	120 to 270 min.	7·2	0·2	4½ hours
60	16	0 to 7 „	1·8	1	3 hours
60	44	7 to 180 „	7·0	0·5	3 hours
60	62	—	Nil	0·3	3½ hours

The Liberation of Adenyl Compounds from the Perfused Liver of the Rabbit.

The toxin in doses of from 2 to 20 mg. causes a specific output of adenyl compounds from the isolated perfused liver of the rabbit. Within 10 minutes after the injection pigment and coagulable protein appear in the perfusate and depigmentation during the ensuing hour or more causes the liver to assume a reticulated appearance. The results of a number of experiments are summarized in Table 4.

In these the perfusate was collected over a boiling water bath, but despite this the total output from the organ was invariably much less than the loss estimated by comparison of an extract of the perfused lobe with that of a control sample of liver. The last three experiments in the Table show that the injury to the tissues caused by the toxin was mainly specific, neutralized toxin causing a relatively small loss of adenyl compounds from the organ. In these control experiments there was no depigmentation and only traces of coagulable protein and adenyl compounds were set free into the perfusate.

After the injection of unneutralized toxin the output of adenyl compounds commenced during the first 10 minutes and continued for three hours; it reached a maximum during the first hour and then diminished gradually. For example, in the experiment in which 20 mg. of toxin were injected intraportally into a lobe of liver weighing 12·6 gm. there was no detectable activity in the samples obtained during half-an-hour before the injection. In the first three minutes after the injection adenyl compounds equivalent to 0·03 mg. adenosine, during the next 7 minutes 0·25 mg., during the next 20 minutes 1·18 mg. and during the second half-hour adenyl compounds equivalent to 0·68 mg. adenosine were estimated in the perfusate. The amounts for the following half-hour samples were equivalent to 0·14, 0·08, 0·1 and 0·1 mg. adenosine respectively. A control extract of liver contained adenyl compounds equivalent to 1 mg. of adenosine per gm. and an extract of the perfused lobe which now weighed 16 gm. contained less than the equivalent of 2γ adenosine per gm.

TABLE 4.

Liberation of adenyl compounds from the perfused liver of the rabbit.

Dose of toxin. in mg.	Content of liver in mg. adenosine per gm.	P.c. loss by difference of organ extracts.	Output in perfusate in mg. adenosine.	Output in perfusate as p.c. of total content of tissue.
2	1	50	0·53	3·7*
5	0·75	80	1·5	17
10	1·5	76	0·95	7·6
20	1	100	2·56	20·3
20 (neutralized)	0·83	0	Nil	0
10 (neutralized)	1	16	Nil	0*
10 (neutralized)	0·9	30	0·23	4*

* In these experiments traces of adenyl compounds were present in the perfusate before the injection of toxin.

The toxin not only sets free adenyl compounds but also liberates an agent capable of inactivating them. This was shown as follows. Perfusate was collected at room temperature from a piece of liver for 30 minutes before and for 40 minutes after the injection of 10 mg. of *Cl. septique* toxin. To 40 c.c. of each of these unboiled samples there was added adenosine to make a concentration of 1: 50,000. A portion of each was at once boiled and the remainder incubated at 37° C., samples being removed and boiled after 15, 25, 40 and 60 minutes. There was no loss of adenosine during incubation of the mixture containing perfusate before the injection, the sample boiled after incubation for an hour having the same cardio-depressant activity as that boiled immediately after the addition of adenosine. The perfusate obtained after the injection of *Cl. septique* toxin contained adenyl compounds equivalent to 9γ of adenosine per c.c. before the addition of adenosine. When this mixture was incubated there was no estimable loss of activity during the first fifteen minutes but after 25 minutes it had lost 25 p.c., after 40 minutes 40 p.c. and after 60 minutes 50 p.c. of its activity. In a control experiment perfusate collected during 1½ hours after the injection of 10 mg. of neutralized toxin was found to contain no inactivating agent, there being no loss of activity in a sample made up to contain 1: 50,000 adenosine and incubated for one hour at 37° C. The liberation of inactivating agent by the toxin is therefore wholly specific.

The Action of the Toxin on Smooth Muscle.

(i) *The isolated jejunum of the guinea-pig.* The toxin in doses of 2 to 5 mg. causes a slight immediate relaxation of the gut followed in 15 to 20 seconds by a small contraction. These effects are non-specific. After a latent period of 2 minutes or more there is a strong rather slow specific contraction of the muscle

from which relaxation takes place only gradually, followed by a prolonged period of diminished excitability.

Fig. 8 I at A shows the response to 5 mg. of neutralized toxin and at B the response of the same piece of gut to 5 mg. of unneutralized toxin. The specific response at B which had a latency of 2¼ minutes was superimposed upon the early nonspecific contraction. Fig 8 II shows the feeble response of a piece of jejunum from another guinea-pig to 1 mg. of toxin (at S) left in contact with the gut for 10 minutes. The specific response of the same piece of gut to 4 mg. toxin at S′ is superimposed upon an initial non-specific response. The gut had not relaxed an hour after changing the Tyrode solution and the response to 0·5γ histamine (at H) was then trivial. In Fig. 8 III from another piece of jejunum from the same guinea-pig as Fig. 8 II, the specific response to 2 mg. of toxin at S″ began after relaxation from the non-specific contraction. Relaxation from the specific contraction was incomplete in 80 minutes and the response to 0·1γ histamine was then diminished.

(ii) *The uterus of the virgin guinea-pig.* The toxin in doses from 0·5 to 5 mg. causes a strong slow contraction after a latent period of from 4 to 1½ minutes. Relaxation is very slow, and after a dose of 2 mg. is not complete in 3 hours, when a second dose causes a slightly diminished response. Relaxation cannot be hastened by treating the muscle with excess of antitoxin. In the contracted state the uterus relaxes sharply when adrenalin is added to the bath but on changing the Ringer's solution it again contracts to its former level. Repeated administration of adrenalin does not hasten the rate of relaxation. The response of the uterus to the toxin is specific.

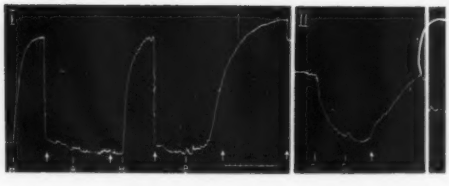

Fig. 9. Responses of a horn of the uterus from each of two guinea-pigs to the toxin of *Cl. septique*. At the arrows the Ringer's solution in the bath was changed. Time in minutes. Details in text.

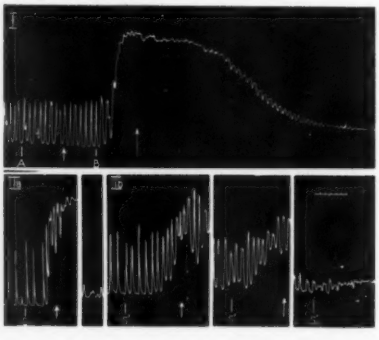

Fig. 10. Responses of 3 horns of the uteri of two virgin rats to the toxin of *Cl. septique*. At the arrows the Ringer's solution in the bath was changed. Time in minutes. Details in text.

Fig. 9 I shows the failure of a uterine horn to react to a dose of 5 mg. of neutralized toxin at A and its response after a latency of 85 seconds to the same dose of unneutralized toxin at B. Each dose was left in contact for 7 minutes. The Ringer's solution in the bath was again changed after 19 minutes and 45 minutes later the muscle had relaxed only to a very slight extent, as shown by the vertical line on the stationary drum at the right of Fig. 9 I. At H are the responses to 5γ histamine left in contact with the muscle for 5 minutes. The latency of each was 5 seconds. Fig. 9 II shows the response of a horn of the uterus of another guinea-pig (2 hours after treatment with 2 mg. of toxin) to 2γ adrenalin at C and to 4γ at D and the return of the muscle to its former level of contraction when the Ringer's solution in the bath was changed after the adrenalin had been in contact with the muscle for 5 minutes. The uterus was incompletely relaxed after 5 hours (right hand panel of Fig. 9 II) and still gave a transitory relaxation to adrenalin.

(iii) *The uterus of the virgin rat.* The toxin in doses of 1 to 10 mg. after latent periods of 8 to 2 minutes causes contraction of the muscle and diminution or abolition of its natural rhythm. Relaxation is slow. While the muscle is con-

tracted adrenalin gives good relaxation but when the Ringer's solution in the bath is changed the uterus at once returns to its contracted state. The administration of adrenalin in repeated doses (1γ) does not hasten relaxation. Histamine in doses of 100γ also causes transient relaxation of the uterus but does not hasten relaxation from the contraction caused by the toxin. The toxin in repeated doses impairs the rhythmicity of the uterus and successive small doses give diminished responses. The response of the uterus to this toxin is specific.

Fig. 10 I shows the failure of a horn of the uterus to respond to 5 mg. of neutralized toxin (at A) left in contact with the muscle for 7 minutes, and the response (at B) to 5 mg. of unneutralized toxin left in contact for the same time. The latency of the response was 2½ minutes and, after 40 minutes, relaxation was not complete and the rhythm greatly diminished. Fig. 10 IIa shows the response to 2 mg. of toxin at S left in contact with the muscle for 6 minutes. The latency was 4½ minutes and relaxation from the contraction took place after 40 minutes, the natural rhythm of the muscle being greatly reduced. Fig. 10 IIb shows the effect of three successive doses of 1 mg. of toxin at S′ on the second horn of the same uterus. The first dose was left in contact for 10 minutes and the reaction had a latency of 7 minutes. Relaxation took place in 35 minutes. The second dose which was also in contact for 10 minutes caused a smaller response and after an interval of an hour the third dose of 1 mg. was left in contact for 15 minutes and failed to cause any response. A dose of 10 mg. after a further interval was also without effect.

DISCUSSION.

The initial non-specific histamine-like effect of the toxin observed in the cat but not in the rabbit is attributable to an impurity. This or some other impurity is responsible for part of the early circulatory disturbances produced by intravenous injection in the cat, manifested by a fall in the systemic pressure and by a rise in the pressure in the pulmonary artery and in the venous pressure. Part of the effects observed upon the coronary circulation of the isolated hearts of the cat and of the rabbit is also non-specific.

Apart from this the toxin has a specific cardiac action indicated in both species by a fall in systemic pressure and a rise in venous pressure, and in the rabbit by a simultaneous fall in pressure in the pulmonary artery. The failure of the heart which occurs when large doses are administered intravenously results partly from direct toxic action on the heart muscle, described by Lautenschläger (1920) and by Buttle and Trevan (1928), and partly from constriction in the coronary and pulmonary vessels, which are probably also direct effects of the toxin and not caused by the liberation of histamine or other muscle-stimulating substance.

In the rabbit, the early rise of systemic blood pressure is not attended by a simultaneous rise in pressure in the pulmonary artery and hence cannot be of directly cardiac origin. Dale attributed it to peripheral vasoconstriction, a view which is supported by the results of the intra-arterial injection of small doses of toxin into the hind limb. In the cat, a rise of systemic pressure is irregularly observed some minutes after the intravenous injection of toxin, but we were unable to demonstrate any constrictor effect by intra-arterial injection into the hind limb.

In the rabbit when recovery occurs from the early cardiac failure after the intravenous injection of moderate doses of toxin there is a steep rise of blood pressure attended by a quickening of the heart beat. The effect resembles that seen in recovery from asphyxia. Since in our experiments the artificial ventilation was adequate and the blood bright throughout it was probably caused by failure of the blood supply to the central nervous system. As the heart recovers, the rise in systemic blood pressure and quickening of the beat occur in the same way and doubtless by the same mechanism as when oxygen is restored after ventilation with nitrogen. That the effect results from a general nervous discharge is indicated by its demonstration in the decapitate preparation, as well as in animals in which constriction in the splanchnic area and the liberation of adrenalin from the suprarenal glands were excluded.

In the cat the final fall of blood pressure is attended or preceded by a rise in pressure in the pulmonary artery and by the development of pulmonary oedema. The pressure in the pulmonary artery stays at a high level and the pulsations in the manometer recording it are maintained while those in the manometer recording the falling systemic pressure are progressively enfeebled. In cats in which the thorax is open it is even possible to observe the development of oedema in the right lung, the ventilation of which has to be increased to remain adequate, and the shrinkage of the left auricle as it gradually becomes empty of blood. In prolonged experiments after moderate doses of toxin, loss of fluid from circulation occurs irregularly and is wholly accounted for by oedema of the lungs. It is possible that in the cat these changes may be exaggerated by the presence in many of the animals of the old lesions of a pneumonia of virus origin which has flourished in this part of Australia for the last two years.

In the rabbit constriction in the pulmonary circulation also occurs regularly and play a part in the final failure of the heart, but oedema of the lung rarely develops.

In the cat the liberation of adrenalin from the suprarenal glands by intra-arterial injection of small doses of the toxin may conceivably be caused by a preliminary liberation of histamine, since the latency of the rise of blood pressure after toxin is longer than after histamine. From the suprarenal of the rabbit, which is much less sensitive to the action of histamine, we were unable to cause any liberation of adrenalin in similar experiments.

The prolonged contraction caused by the toxin in the three varieties of plain muscle which we studied appears to be a direct effect of the toxin itself. We were unable to demonstrate the liberation from the guinea-pig's lungs of any muscle-stimulating substances other than histamine, and histamine itself cannot be held responsible, at least in the rat uterus, in which this substance in relatively large doses causes relaxation.

Buttle and Trevan (1928) showed that after one contraction the smooth muscle of the rabbit's uterus became insensitive to subsequent doses. This effect is exhibited by other poisons and toxins—for example, by snake venoms (Kellaway, 1929), as well as by the toxins of other anaerobes. The duration of contraction caused by the toxin of *Cl. septique*, particularly in the uterus of the virgin guinea-pig, is not attributable to the continued action of the toxin since it is not overcome by treatment with large excess of antitoxin. It is also not wholly attributable to permanent injury of the muscle because the muscle relaxes well after small doses of adrenalin, though it returns to its previous contracted state as soon as the drug is washed away. That there is some directly noxious effect on the muscle is shown by the impaired response to histamine when relaxation occurs after the response of the isolated jejunum of the guinea-pig to toxin. The prolonged contraction cannot be accounted for by the continuous liberation of some muscle-stimulant substance by injury, for we were unable to demonstrate such substance in the perfusate from isolated organs. The long latency of the contraction may possibly be accounted for, as Buttle and Trevan suggest, by the slow diffusion of the large molecule of the toxin into the muscle.

SUMMARY.

The toxins used in these experiments contained impurities causing non-specific effects, depressor on the blood pressure of the cat and dilator in small doses and constrictor in large on the coronary vessels of the cat and rabbit. The liberation of histamine from the perfused lungs of the guinea-pig and of adenyl compounds from the perfused liver of the rabbit were in part non-specific. The isolated jejunum of the guinea-pig after a short latency gave a small non-specific contraction from which relaxation occurred fairly quickly.

In the cat the toxin of *Cl. septique* injected intravenously in large doses causes specific constriction in the coronary and pulmonary circulations. In smaller doses it causes constriction in the pulmonary circulation and oedema of the lungs with consequent loss of fluid from the circulation. The toxin injected intra-arterially into the vessels supplying the suprarenal glands causes liberation of adrenalin. It has no direct constrictor effect on the vessels of the hind limbs.

In the rabbit the initial rise in blood pressure is caused by direct peripheral vasoconstriction. The subsequent fall of systemic blood pressure, accompanied by fall of the pressure in the pulmonary artery and rise in the venous pressure can be accounted for by the direct effect on the cardiac muscle (demonstrated by Lautenschläger and by Buttle and Trevan) reinforced by constriction of the coronary vessels. There is also constriction in the pulmonary circulation, but pulmonary oedema is inconstant. The effects on the systemic circulation are observed to be unaltered after section of the splanchnic nerves, after decapitation and after evisceration with excision of the suprarenal glands. The toxin injected intra-arterially causes transient specific constriction of the vessels in the hind limb.

The toxin causes the liberation of histamine from the isolated perfused lungs of the guinea-pig and of pigment and of adenyl compounds together with an inactivating enzyme from the perfused liver of the rabbit.

It causes specific contraction with long latency and slow-relaxation in the isolated jejunum of the guinea-pig and the uteri of the virgin guinea-pig and rat.

REFERENCES.

Buttle, G. A. H. and Trevan, J. W. (1928): Brit. J. exp. Path., 9, p. 182.
Dale, H. H. and Laidlaw, P. P. (1919): J. Physiol., 52, p. 355.
Drury, A. N., Lutwak-Mann, C. and Solandt, O. M. (1938): Quart. J. exp. Physiol., 27, p. 215.
Feldberg, W. and Kellaway, C. H. (1937): J. Physiol., 90, p. 257.
Feldberg, W. and Kellaway, C. H. (1938): *Ibid.*, 94, p. 187.
Feldberg, W. and Schilf, E. (1930): Histamine, Springer, Berlin, p. 229.
Gunn, J. A. (1913): J. Physiol., 46, p. 506.
Kellaway, C. H. (1929): Brit. J. exp. Path., 10, p. 281.
Kellaway, C. H. and LeMessurier, D. H. (1936): Austral. J. exp. Biol., 14, p. 57.
Lautenschläger, I. L. (1920): Arch. f. exp. Path., 85, p. 1.
Robertson, M. (1919): J. Path. Bact., 23, p. 162.
Straub, W. (1919): Munchen med. Wchnschr., 66, p. 89.

THE DETERMINATION OF GLYCOGEN IN OYSTERS

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(Accepted for publication 3rd September, 1941.)

The method of determination of glycogen here described was devised by selecting those parts of the methods of various workers on this problem which tests indicated as most suitable for the particular purpose in view.

THE METHOD.

The sample immediately on opening is strained through cheese cloth and the weight of the wet meat determined. It is then placed in a bottle with 2 ml. of 60 p.c. KOH per gm. of wet meat. The sample on receipt at the laboratory is transferred to a volumetric flask and heated in the boiling water bath for two hours, swirling occasionally for the first few minutes; it is then allowed to cool and made up to volume.

Duplicate 5 ml. aliquots are transferred to 50 ml. pyrex centrifuge tubes which are then heated in the boiling water bath and 6 ml. of 95 p.c. alcohol added; after mixing, the contents are heated to boiling. On cooling the tubes are spun for 10 minutes at about 2,500 r.p.m. The supernatant liquor is then decanted and the residue redissolved in 5 ml. of water by heating in the water bath. The glycogen is reprecipitated with alcohol as before and, after spinning and decanting, 10 ml. of 5N sulphuric acid are added and the solution heated in the boiling water bath for half an hour, the tubes being covered with small filter funnels.

The solution is next transferred to a 250 ml. volumetric flask and neutralized with 40 p.c. sodium hydroxide. After making up to the volume the sugar is determined by the Shaffer-Somogyi cuprous method (1933), using reagent 50.

The factor 0·927 is used to convert glucose into glycogen.

EXPERIMENTAL.

Extraction of glycogen. The liquor in the shells was discarded as analyses showed that it contained no significant amount of glycogen; this is in agreement with the findings of Mitchell (1915–16) but contrary to those of Tully (1936).

Digestion. In the following experiment the samples were heated for one hour, cooled, made up to the volume and aliquots removed for analysis. The remainder was heated one hour more, cooled, etc. The glycogen content was then calculated after allowing for the fact that some of the material had been removed in the aliquots used for previous estimations.

Sample.	Time in hours.	Percentage glycogen.
1	1	4·08
	2	3·81
	3	3·81
2	1	5·24
	2	4·77
	3	4·84
3	1	5·72
	2	4·98
	3	5·05

Therefore two hours' heating was used in determinations.

Precipitation of glycogen. Various workers, Bell and Young (1934), Good *et al.* (1933) and Kerr (1936) have used different methods for precipitating glycogen. The glycogen here was precipitated by 1·2 volumes of alcohol; no reducing substance was found in the mother liquor after distilling off the alcohol, and heating for half an hour in the boiling water bath in the presence of 5N sulphuric acid.

Hydrolysis of glycogen. The glycogen in a potash digest, after two precipitations, was hydrolyzed by 15 ml. of 0·6 N HCl for three hours, by 15 ml. of N HCl for two hours, or (Sahyun 1933), 10 ml. of 5N H_2SO_4 for half an hour.

The three methods yielded similar results.

	N HCl.	0·6N HCl.	5N H_2SO_4.
Glycogen†	100	103	99
	100	100	104
	100	100	100

† Although three different digests were used the figures are adjusted to give, in every case, the value 100 for hydrolysis with N HCl.

Precipitates in the acid hydrolysates and neutralysates. Except where pure glycogen solutions were used, black mud-like precipitates were noted in the acid hydrolysates. This did not interfere with the precision of the sugar method but it was feared that it might affect its accuracy. Tully (1938), using the Shaffer-Hartman cuprous method for sugar determination said that the sugar solution, i.e. the neutralysate, must be filtered before determination. He gave the result of only one estimation however.

The following experiments were therefore carried out. The precipitates in the acid hydrolysates were filtered off and washed four times with 15 ml. lots of N HCl, since N HCl was used for hydrolysis. The neutralysate was not filtered; unfiltered hydrolysates were used as controls.

	Filtered.	Unfiltered.		Filtered.	Unfiltered.
Glucose (mg.)	1·40 ·74	1·41 ·73	Glucose (mg.)	·38 1·03	·38 1·03

The following were obtained by filtering the neutralysates but not the hydrolysates.

	Filtered.	Unfiltered.		Filtered.	Unfiltered.
Glucose (mg.)	·73 ·80	·72 ·80	Glucose (mg.)	·97 ·96	·96 ·95

The following were obtained by filtering both, using both unfiltered as controls.

	Filtered.	Unfiltered.		Filtered.	Unfiltered.
Glucose (mg.)	·52 1·16	·51 1·17	Glucose (mg.)	1·17 1·69	1·17 1·72

Accuracy of the method. To estimate the extent of glycogen loss under conditions approximating to experimental, the following experiment was carried out. To two 250 ml. volumetric flasks, each containing 10 gm. of egg albumen (B.D.H.), 3 gm. of lard (Foley's), 80 gm. KOH, 15 mg. $CuSO_4.5H_2O$, 100 mg. $ZnSO_4.7H_2O$, 1·2 gm. NaCl, 400 mg. $MgSO_4.7H_2O$, 15 mg. $FeCl_3$, 500 mg. $CaCl_2$ and 1·0 gm. KH_2PO_4 were added 50 and 100 ml. respectively of glycogen solution. These salt concentrations were taken from the following papers: McCance and Shipp (1933), Rose and Bodansky (1920) and Bodansky (1920).

The volumes were made up to about 200 ml. and the flasks heated in the boiling water bath for two hours. At the same time 25 ml. of the same glycogen solution were heated with about 20 ml. of water under the same conditions. The glycogen was then determined in the solutions after making up to the mark.

	Added.	Recovered.
Glycogen (gm.)	1·78 3·56	1·68 3·50

Attempts were also made to recover glycogen added to KOH digests. In all cases the glycogen and the digest were heated together for two hours.

	Before addition.	Added.	Recovered.	Percentage recovery.
Glycogen (mg.)	17·8	46·7	45·5	98
	19·5	17·5	17·0	97
	56·2	23·9	22·7	95

It is seen that the greatest loss was 5 p.c. The accuracy of the method may not however be stated as 5 p.c. since the glycogen was not liberated from combination as it might be in the case of the "desmo-glycogen" in oysters (Bancroft and Bancroft, 1931).

SUMMARY.

A method is described for the determination of glycogen in oysters. The various features of the procedure have been investigated.

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REFERENCES.

Bancroft, W. D. and Bancroft, D. (1931): J. phys. Chem., 35, p. 194.
Bell, D. J. and Young, F. G. (1934): Biochem. J., 28, p. 882.
Bodansky, M. (1920): J. biol. Chem., 44, p. 399.
Good, C. A., Kramer, H. and Somogyi, M. (1933): *Ibid.*, 100, p. 485.
Kerr, S. E. (1936): *Ibid.*, 116, p. 1.
McCance, R. A. and Shipp, H. L. (1933): Med. Res. Counc. Spec. Rep. No. 187.
Mitchell, P. (1915–16): Bull. U.S. Bur. Fish., 35, p. 135.
Rose, W. and Bodansky, M. (1920): J. biol. Chem., 44, p. 99.
Sahyun, M. (1933): *Ibid.*, 103, p. 203.
Shaffer, P. A. and Somogyi, M. (1933): *Ibid.*, 100, p. 695.
Tully, J. P. (1936): J. Biol. Bd. Can., 2, p. 477.
Tully, J. P. (1938): Analyst, 63, p. 93.

RELATIONS BETWEEN RESPIRATION RATE AND METABOLISM OF CARBOHYDRATE, PROTEIN, AND ORGANIC ACIDS IN LEAVES

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In this paper we have attempted to assess relations between respiration rate of leaves, both in air and in nitrogen, and total and cytoplasmic protein; and also, throughout the life cycle of a grass plant, to investigate the relations between respiration rate and carbohydrates, amino-compounds and organic acids.

NOTATION.

To conserve space the following notation has been adopted: P, protein-nitrogen; A, residual amino-acid nitrogen; S, sucrose; OR, respiration rate in air; NR, respiration rate in nitrogen. The following subscripts to any of the above symbols indicate the basis on which they have been calculated: w, dry weight basis; p, total protein basis; c, cytoplasmic protein basis.

EXPERIMENTAL.

Material. Plants of a pure line of Sudan Grass (*Andropogon sudanensis*, L. and B.) were grown in pots containing 14 kg. of a mixture of sand and loam. Seeds were planted on 26th February, 1940, and exsertion of inflorescences commenced on 24th April. The loam had a N-content equivalent to 8·5 gm. $NaNO_3$ per pot. The following nutrients were added to each pot 7 days after planting: 0·4 gm. $NaNO_3$; 0·6 gm. KH_2PO_4; 2·0 gm. K_2SO_4; 0·4 gm. $MgSO_4.7H_2O$; 0·74 gm. $CaCl_2$; 0·06 gm. $FeCl_3$; 0·0016 gm. $MnSO_4.4H_2O$.

The plants were divided into two Series. Harvests were made in Series I, 10, 23, 35, 43, 51, 58, 71 and 85 days after planting; in each case harvests were made at 8.30 a.m. Harvests on days 23, 35, 51, 58, 71 and 85 correspond to harvests 1, 2, 3, 4, 5 and 6 of a previous paper by Hanson, Barrien and Wood (1941) wherein relations between chlorophyll, total protein and chloroplast protein were described. In Series II, 1 gm. $(NH_4)_2SO_4$ was added to each pot 24 hours prior to harvest. It was hoped in this way to increase the protein content of the leaves; this expectation was not realized as the time was insufficient; the only significant changes in Series II compared with Series I are in amino-acid-N content. In effect therefore Series II serves as a check on the values obtained in Series I. Harvests of Series II were made one day after those of Series I.

Owing to the large amounts of leaf material required for the estimation of chloroplast protein it was not possible to use uniform ontogenetic material at each harvest. Each harvest therefore, included the whole of the leaves, cut at the ligule, from an equal number of plants.

Respiration rate and chloroplast-protein were determined in fresh material; other substances were determined on material rapidly dried in an air current at 90° C.

Respiration rate. 20 gm. fresh leaves were used in each case and placed in black containers in a water bath at 24·60 ± 0·02° C. CO_2 produced by the leaves was absorbed in N/10 NaOH and determined by changes in electrical conductivity. The gas streams were maintained at 7 litres per hour by means of appropriate needle valves and manometers. Air was obtained from outside the laboratory by means of a pipe line and before entering the respiration chambers was passed through soda-lime towers and water bubblers. The absorbing cell had a high constant and consisted essentially of a pyrex tube containing fixed electrodes and connected at the top and bottom to a spiral tube which surrounded the central vessel and into which the air stream containing CO_2 entered by means of an injector. 40 ml. NaOH solution were used on each occasion.

We used an A.C. bridge, including a specially designed valve oscillator of 1,000 cycles output; resistance box, with ratio arms, accurate to 1: 20,000 ohms; a two stage radio amplifier and a Wagner earth. A cathode ray tube was used as null-point instrument and proved more sensitive than high resistance piezo-electric phones. Recordings could be made with ease at 5 min. intervals. The NaOH was calibrated in terms of CO_2 by using standard NaOH–Na_2CO_3 mixtures.

At each harvest leaves were placed in the chambers ½ hour after cutting; temperature adjustment was rapid since air temperatures were not far removed from that of the bath. Disturbances similar to those described by Audas (1935) occurred at the commencement of readings but after about 1½ hrs. values which decreased steadily over a period of many hours were obtained.

Usually 3 hrs. after placing in the apparatus the air stream derived from the blower was replaced by cylinder nitrogen passed over copper spirals in an electric furnace and cooled to bath temperature. No increase in respiration rate above that in air occurred in nitrogen at any

harvest but values fell rapidly from the previous value in air to a value from which it only slowly decreased with time. In several cases when steadily decreasing values had been obtained nitrogen was replaced by air. This brought about a very rapid rise in respiration rate, presumably due to accumulated alcohol, followed by a fall to values continuing those of the air line prior to entry into nitrogen.

We have taken the value three hours after placing in the apparatus as the measure of *OR*. The line connecting the steadily declining values in nitrogen has been extrapolated back to this three-hour point, i.e. the time of entry into nitrogen, and taken as the value for *NR*.

Analytical methods. Total protein-N and chloroplast protein-N were determined by methods previously reported (Hanson, Barrien and Wood, 1941). Cytoplasmic protein-N is recorded as the difference between the above two protein fractions. Ammonia-N was determined in aliquots of the protein-free filtrate by distillation under reduced pressure with a soda-borate buffer at pH 7·5 and subsequent nesslerization with the Folin-Koch reagent. Glutamine was determined by hydrolyzing aliquots at pH 6·5 with a phosphate-borate buffer, and total amides by hydrolyzing with H_2SO_4 at pH 1; in both cases the ammonia liberated was determined as above. Asparagine is reported as the difference between total amide and glutamine. Total amino-N was determined in a micro-Van Slyke apparatus and the residual α-amino acid-N is obtained by deducting the amino-N of the amides from total amino-N.

Sugars were estimated by the method of Hagedorn and Jensen as outlined by Amos and Wood (1939).

Organic acids were extracted according to the method of Pucher, Vickery and Wakeman (1934); malic acid was determined by the method of Vickery and Pucher (1933) using a Hilger polarimeter; citric acid was determined by the method of Pucher, Vickery and Wakeman (1934). We recommend the use of hexane instead of petrol-ether, which contains unsaturated hydrocarbons, in this method.

All analyses were performed on duplicate samples of the original material and the results reported are means of these values.

RESULTS.

Results of analysis are presented on a dry weight basis in Table 1. Significant correlation coefficients and regression equations are presented in Table 2. Drifts with time of reactants pertinent to the discussion are shown in Fig. 1.

TABLE 1.

Analytical data.

Harvest.	Days after planting.	Mean dry weight per plant.	Water.	Respiration rate in air.	Respiration rate in nitrogen.	Total protein-N.	Chloroplast protein-N.	Cytoplasmic protein-N.	Ammonia-N.	Glutamine-N.	Asparagine-N.	Total amino-N.	Residual amino-N.	Malic acid.	Citric acid.	Oxalic acid.	Hexoses.	Sucrose.	*OR*/*NR* Ratio
Series I.																			
1	10	·0057	710	10·55	5·75	3·00	—	—	—	—	—	—	—	—	—	—	—	—	1·84
2	23	·24	513	5·02	2·30	4·28	·30	3·98	·024	·026	·053	·100	·061	10·6	4·8	0·6	1·36	0·95	2·18
3	35	·88	476	3·24	1·73	3·61	1·4	2·21	·018	·020	·017	·076	·057	8·4	3·6	0·5	1·27	5·65	1·86
4	43	2·02	421	2·82	1·60	3·26	1·26	2·00	·017	·020	·048	·074	·048	12·6	2·6	1·8	1·95	3·01	1·76
5	51	2·40	373	2·84	1·71	2·54	0·91	1·63	·018	·012	·021	·148	·131	11·6	4·3	1·4	2·93	9·89	1·66
6	58	3·46	351	2·14	1·56	2·20	0·90	1·30	·011	·017	·047	·100	·068	10·6	4·1	1·6	2·46	5·18	1·37
7	71	3·63	296	2·36	1·58	1·64	0·74	0·90	·012	·009	·015	·110	·098	17·4	6·2	2·6	3·67	7·16	1·49
8	85	3·57	252	1·78	1·27	1·17	0·49	0·68*	·011	·018	·025	·119	·098	18·8	9·6	5·5	4·38	6·10	1·40
Series II.																			
3a	36	·89	426	3·93	1·64	3·28	1·34	2·94	·020	·013	·013	·143	·130	9·6	2·9	2·5	2·65	6·26	2·39
4a	44	1·97	411	3·54	1·80	3·21	—	—	·020	·027	·031	·177	·148	10·6	4·9	3·8	2·99	4·83	1·96
5a	52	2·40	366	3·50	1·98	2·68	0·94	1·74	·018	·026	·015	·146	·126	11·7	3·4	2·6	3·28	8·06	1·77
6a	59	3·28	340	2·98	1·66	2·04	—	—	·017	·014	·033	·064	·041	10·6	3·8	2·2	3·01	5·00	1·79
7a	72	3·30	306	2·66	1·60	1·92	—	—	·011	·012	·009	·081	·070	21·1	5·7	2·7	2·90	6·69	1·66
8a	86	3·56	253	1·73	1·24	1·12	—	—	·020	·004	·019	·101	·089	25·5	10·7	4·4	3·02	4·51	1·39

* Approximate figure only since chloroplasts disintegrated.

Mean dry weight in gm. per plant.
Respiration rate in mg. CO_2 per gm. dry weight per hour.
Malic, citric and oxalic acids in milli-equivalents per 100 gm. dry weight.
All other fractions in gm. associated with 100 gm. dry matter.

The relations shown to exist between variables in the following discussion suggest that in slow ontogenetic drifts the amounts of reactants may not be far removed from those characterizing a steady state. But this is not so when an additional reactant is suddenly introduced, as ammonia in Series II. The work of Walkley (1940) has shown that under such conditions there is a rapid increase in soluble nitrogen in leaves but that protein is synthesized slowly and does not reach a maximum until about 6 days after application. A similar state of affairs may be seen in the experiment of Amos and Wood (1939). For this reason the results of Series II cannot be directly compared with those of Series I, especially where comparisons are made on a protein basis. Series II provides however useful information on the effect of amino acids on respiration rate and serves as a check on the values of each substance in Series I.

TABLE 2.

Statistical data.

1. *Correlation coefficients:* U, water content on dry weight basis; Pt, protein-N on dry weight basis; Pc, cytoplasmic protein-N on dry weight basis; OR_w, respiration rate in air on dry weight basis; A_w, residual amino-acid-N on dry weight basis; Gl., glutamine-N on dry weight basis; $Asp.$, asparagine-N on dry weight basis. The following symbols are used for substances on a cytoplasmic protein-N basis: OR_c, respiration rate in air; NR_c, respiration rate in N; A_c, residual amino-acid N; S_c, sucrose; C, citric acid; M, malic acid. **P** is the probability that a correlation should arise by chance from an uncorrelated population.

	r	P		r	P
OR_w, Pt	+ 0·896	$<$·01	A_c, C	+ 0·918	$<$·01
OR_w, Pc	+ 0·980	$<$·01	A_c, M	+ 0·942	$<$·01
U, Pt	+ 0·996	$<$·01	OR_c, A_c	+ 0·954	$<$·01
U, Pc	+ 0·921	$<$·01	OR_c, S_c	+ 0·946	$<$·01
A_w, Gl	− 0·659	$<$·1$>$·05	OR_c, C	+ 0·888	$<$·01
A_w, Asp	− 0·547	$<$·2$>$·1	OR_c, M	+ 0·954	$<$·01
S_c, A_c	+ 0·979	$<$·01	NR_c, A_c	+ 0·854	$<$·02$>$·01
S_c, C	+ 0·840	$<$·02$>$·01	NR_c, S_c	+ 0·944	$<$·01
S_c, M	+ 0·885	$<$·01	NR_c, C	+ 0·896	$<$·01
			NR_c, M	+ 0·954	$<$·01

2. *Regression equations:* Symbols as before. Significant regression coefficients are shown in italics. V is the percentage of the variance of OR_c ascribed to the average effect of the independent variables.

	V
(a) $OR_c = 110{\cdot}4 + 783{\cdot}6\,A_c + 4{\cdot}86\,S_c$	85·5
(b) $OR_c = 111{\cdot}3 + \mathit{1106{\cdot}6}\,A_c$	89·3
(c) $OR_c = 110{\cdot}4 + \mathit{16{\cdot}16}\,S_c$	89·6

DISCUSSION.

Aerobic Respiration and its Relation to Other Variables.

Gregory and Sen (1937) and Richards (1938) studying the effect on plants of different degrees of deficiency in nitrogen, potassium and phosphorus have shown that throughout the life cycle, respiration rate is related to both sugar and amino-N content. Their experimental conditions were such that (1) high sugar was associated with low amino content and (2) high amino content with low sugar content. Similar results were reported by Amos and Wood (1939) who supplied varying amounts of N to plants at a definite phase in the life cycle. Both Richards, and Amos and Wood considered that the relation between respiration rate and substrates was of hyperbolic form usual in enzyme actions to which the Michaelis equation applies, and that consequently changes in amounts of one variable produced marked effects when the other was near the asymptote. Barker (1936) has shown that in potatoes stored at low temperatures the relation between OR and sucrose content is hyperbolic.

In certain instances Gregory and Sen (*loc. cit.*) found that OR_w was correlated with total-P_w. In series of plants with a high level of N-manuring the curve relating OR_w to total-P_w was convex to the P-axis whilst in those with a lower level of N-manuring the convexity was decreased. Gregory and Sen concluded that total-P was an unsatisfactory basis on which to compare respiration rates of plants variously manured. Richards, on the contrary, found a high linear correlation between R_w and total-P_w in plants deficient in potassium and phosphorus. Since over the whole treatment range both amino-N and sugar contents had marked effects he considered that the simple concept of P_w as a measure of the number of respiratory seats was insufficient to

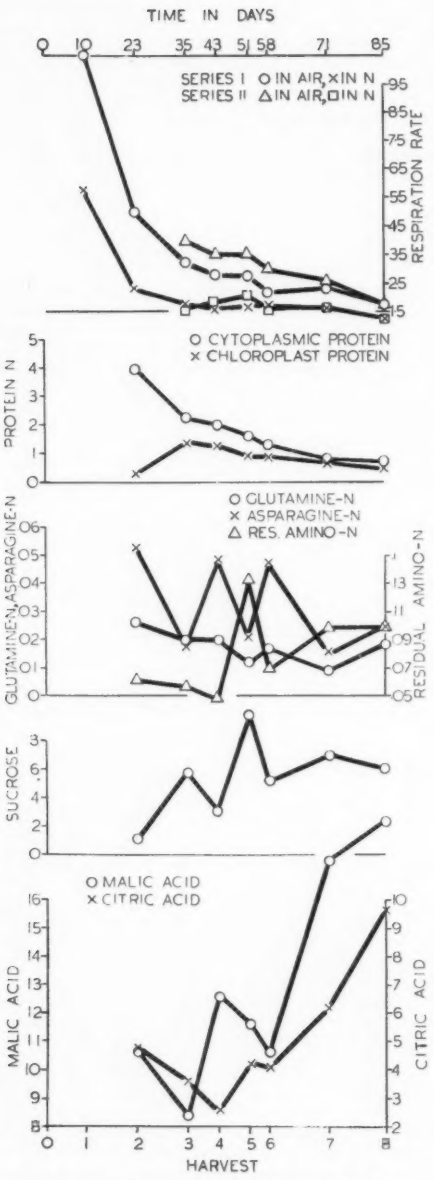


Fig. 1. Reactants on dry weight basis plotted against time. Series I. Respiration rates in mg. CO_2 per gm. dry weight per hour; malic and citric acids in milliequivalents per 100 gm. dry weight; other substances in gm. per 100 gm. dry weight.

account for the data and suggested a reciprocal relation between P_w and OR_w. Petrie and Williams (1938) presented data for Sudan grass and oats over a wide ontogenetic range which indicates a close correlation between OR_w and P_w but again the curve is convex to the P-axis. They suggested that total protein-N was a measure of amount of cytoplasm, and that the decline in OR_w with time was largely the result of variations in cytoplasm content.

If the values for OR_w of the above-mentioned authors are placed on a protein basis and plotted against time it is apparent that in plants manured at a high N-level, OR_p tends to remain relatively constant, throughout the life cycle, although it may increase or decrease slightly. On the other hand, OR_p increases with time when the level of N-manuring is lowered; the greater the deficiency in N, the sooner does OR_p increase (Gregory and Sen; Petrie and Williams).

Gregory and Baptiste (1936) have described the leaves of the N-deficient plants as pale green in colour, indicating low chlorophyll development. It appeared possible therefore that in N-deficiency total protein-N might approximate to cytoplasmic content, whilst in plants with a high level of N-manuring other storage proteins, which might not be respiratory centres, would be present in relatively large amount. Such a hypothesis would account for differences observed in OR_p with time in plants variously manured. Accordingly in this experiment we have determined both total protein-N and chloroplast protein-N. The difference between these two values we have termed "cytoplasmic-N".

Chloroplast protein behaves in many respects as a storage protein; it differs from cytoplasmic proteins in its gel state; the present experiment shows that amount of chloroplastic protein is very low in young leaves, but later rises to a maximum and thereafter declines; it is known that chlorophyll disappears rapidly during starvation; Hanson (1941) has shown that chloroplast protein increases together with cytoplasmic-N when plants are manured with additional N throughout the life cycle and also that during senescence chloroplast-protein disappears more rapidly than does total protein; Hanson, Barrien and Wood (1941) have shown in Sudan Grass that chloroplast protein differs from cytoplasmic protein in that the former is richer in protein-S than the latter. It cannot be said with certainty that chloroplasts do not respire but the linearity of the OR_w/cytoplasm-N relation described below suggests that P_c offers a more likely measure of the number of respiratory seats than does total protein.

In Fig. 2b both OR_c and OR_p are plotted against time. OR_c rises markedly with time as is the case of the N-deficient series of Gregory and Sen (1937) and of Petrie and Williams (1938), whilst OR_p rises slightly towards the end of the life cycle and approximates to the form of OR_p in the fully manured plants of the authors cited.

OR_w gives significant correlations with both total and cytoplasmic protein on a dry weight basis but the correlation is greater with cytoplasmic protein (See Table 2). The higher correlation of OR_w with cytoplasmic protein depends in large measure on the value determined for chloroplast protein in Harvest 2. We would point out that this value is the mean of four closely agreeing replicate samples. In Fig. 2a OR_w is plotted against total- and cytoplasmic protein-N In the latter case the relation between variables is a linear one whilst with total protein the relation between variables is more complex and suggests that relatively greater respiration is associated with unit protein at later stages in the plant's development (low total protein) than at earlier stages (high total protein).

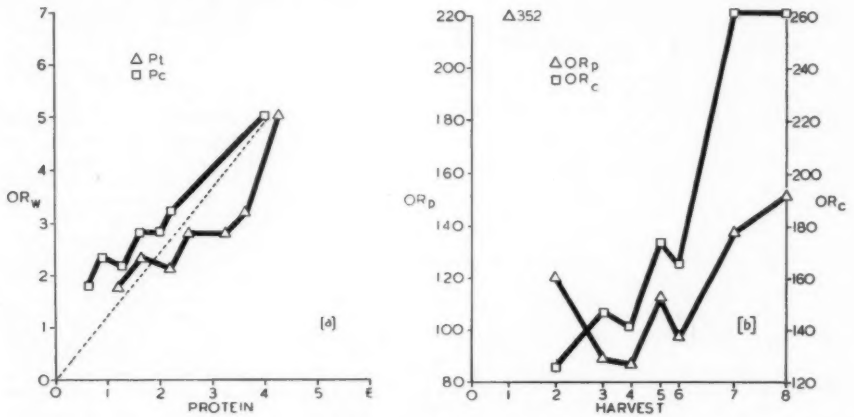


Fig. 2. (a) Respiration rate in air (mg. CO_2 per gm. dry weight per hour) plotted against total protein-N and cytoplasmic protein-N (in gm. per 160 gm. dry weight). (b) Respiration rate in air on a total protein-N basis and on a cytoplasmic protein-N basis (mg. CO_2 per gm. protein-N per hour) plotted against time. The value of OR_p for harvest 1 is not drawn to scale and consequently not joined to the value for harvest 2; the correct value is 352.

It will be shown below that fluctuations in the OR_w/cytoplasmic-N curve are correlated with variations in A and S.

It can be seen in this experiment, as in those of Gregory and Sen, Richards, and Petrie and Williams, that the curve expressing the OR_w/P relation does not pass through the origin. In Fig. 2a a dotted line is drawn through the origin and joins the value for Harvest 2 where cytoplasmic protein is approximately equal to total protein and where S and A are both low, i.e. it represents respiration rate at constant substrate level but with varying cytoplasmic content which we may assume is equivalent to varying respiratory seats. The experimentally determined values for OR_w all lie above this line and represent the effects of varying S and A.

Before considering the relations between OR_c and substrates we draw attention to certain features in Fig. 2b wherein OR_c is plotted against time. Respiration rate falls from a high level at the seedling stage (and where the high respiratory level is presumably due to substrates derived from endosperm) to a low value from which it gradually rises to a maximum during senescence (yellowing) of the leaves. We would point out the formal resemblance of the course of respiration rate on a cytoplasmic basis plotted against time with that of respiration rate on an absolute basis of storage organs (such as apples and potatoes) under starvation conditions described by Blackman and Parija (1928) and Barker (1936). The resemblance is not surprising for in the storage structures mentioned it is probable that cytoplasmic content undergoes little change and Barker (*loc. cit.*) has shown that respiration rate is determined mainly by sucrose content. In effect respiration of such organs and amount of substrates are studied upon a constant cytoplasm basis.

We have therefore examined the relations which exist between respiration rate and other variables all expressed on a cytoplasm-protein-N basis. This procedure possesses the additional

advantage that in effect it places reactants upon a concentration basis for in the experiment described here water content and protein content are highly correlated throughout the life cycle (see Table 2).

In Table 2 it can be seen that OR_c, is highly correlated with A_c, S_c, malic acid and citric acids; and also that the latter four variables are highly correlated with one another. The regression equation of OR_c on A_c and S_c (Table 2) accounts for 85 p.c. of the total variance but neither regression coefficient is significant; as might be expected, if either A_c or S_c is eliminated from the equation the resulting regression is highly significant and accounts for 90 p.c. of the variance.

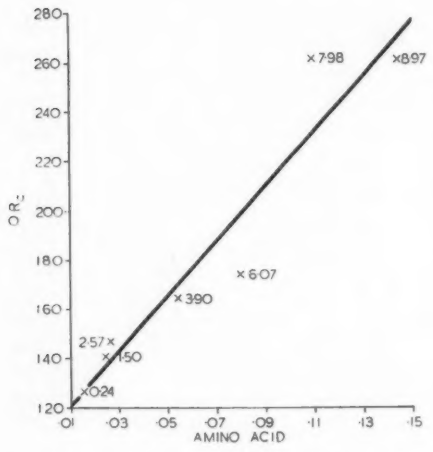


Fig. 3. Respiration rate in air on a cytoplasmic protein basis plotted against residual amino-acid content on the same basis. The regression line is that of Equation (b) in Table 2. The corresponding value of S_c is given for each point.

Gregory and Sen (*loc. cit.*) also found A and S to be highly correlated in their experiment, but in Richards's experiment A was correlated not with sucrose but with hexoses.

In Series II where A and S are not correlated the effect of additional A on OR is marked (see Fig. 1).

When OR_c is plotted against either A_c, S_c, citric or malic acids the curve is possibly a rectangular hyperbola but all the experimental values lie on an approximately straight line on the upper part of the curve (Fig. 3).

On the other hand when citric or malic acids on a cytoplasmic basis are plotted against either A_c or S_c the regression line passes close to the origin.

It is apparent therefore that any scheme must take into account the high degree of correlation between respiration rate, amino-acids, sucrose, citric and malic acids: such a scheme is suggested in the last section of this discussion.

There remains to consider the position of amides. A noticeable feature in this experiment is the inverse relationship apparent between both amides on the one hand and with amino-acids and respiration rate on the other (see Fig. 1). The correlation coefficient of A_w on glutamine is barely significant at the 5 p.c. point and that of A_w on asparagine is significant only at the 10 p.c. point.

Wood and Petrie (1938) in Experiment I of that paper have reported a similar result wherein the drifts of amide with time are in the reverse direction of those for amino-acid content and respiration rate. There is no doubt from the experiments of Wood and Petrie (*loc. cit.*) that amides increase together with amino acid content and respiration rate when additional nitrogen is supplied to the plants.

The results suggest that amides increase in amount as respiration rate increases and provides more substrate for synthesis but superimposed on this relation is the possibility that a steady state relationship exists between amino-acids and amides which is determined at each point by the respiration rate and in such a direction that with increased respiration rate the position of equilibrium is pushed towards the side of amino-acid formation.

Anaerobic Respiration and its Relation to Other Variables.

When air is replaced by nitrogen the values for respiration rate do not rise above the rates in air as described for apples by Blackman and Parija (1928) and in some cases for carrot tissue by Turner (1940). On the contrary the values fall rapidly from the previous value in air to a value from which they decrease only slowly with time. The period of rapid fall was complete in about an hour in all cases.

Values of the ratio Final OR/Initial NR in all cases are less than the theoretical value of 3, supposing hexose to be the sole substrate. The values for the ratio also tend to decrease with increased age of the leaves. The departure from the theoretical ratio is usually ascribed to oxidative anabolism in air. Such a mechanism may be operative here, but it is also possible that in the experiment described here the observed departures may be related to increased utilization of amino-acid in later stages of the life cycle. Our experiments were not designed to elucidate these points and further discussion is unprofitable at present.

NR_w plotted against protein exhibits the same characteristics as does OR_w i.e. a linear relationship with cytoplasmic-N, the fluctuations being accounted for in terms of sucrose and amino-acid contents. The NR_w/P_w curve is convex to the P-axis as in the case of OR.

NR_c, like OR_c, shows high linear correlations with S_c, A_c and citric and malic acids on a cytoplasmic basis (see Table 2). It is noticeable however that the value for the coefficient of NR_c on S_c is higher than that of NR_c on A_c, whereas in the case of OR_c the correlation coefficients were approximately equal with both A_c and S_c.

Series II indicates, as in the case of OR, that amino-acids may be substrates in NR but the percentage increase in NR above that in Series I is not so great at each harvest as the percentage increase in OR above that in Series I. This suggests a greater utilization of amino-acid in OR than in NR.

Reciprocal Relations between Respiration Rate and Proteins.

From the foregoing discussion it would seem that in this experiment respiration rate is accounted for in terms of cytoplasmic-N (which may be a measure of number of respiration centres) sucrose and amino-acid N. There remains to consider the possibility of reciprocal relationships, especially between total protein and respiration rate. Such relations have been suggested by Richards (1938) and imply that a certain respiration rate is necessary to maintain a definite amount of protein; at a steady state the rate of protein hydrolysis would be balanced by formation of an amount of P proportional to OR.

If we examine Fig. 2a each point on the dotted line joining the origin to the value for Harvest 2, may be regarded as the amount of cytoplasmic protein determined by a definite respiration rate; the experimentally determined values (where sucrose and amino-acids vary in amount) lie above this line and their position above the line can be looked upon as a measure of excess energy which can be utilized in building fresh protein. If then OR is plotted against total protein we should obtain a line lying below the dotted line and a mirror image of the OR/cytoplasmic protein curve; that is, fluctuations due to substrate should be reflected in reverse manner to those of OR plotted against cytoplasmic-N. In the early harvests (high protein values) this supposition is borne out, but there is a divergence from linearity in the later harvests. The data of Gregory and Sen (1937) and of Petrie and Williams (1938) also show a similar convexity in the OR/P curve.

It would appear therefore that the simple concept of total protein maintained at all stages by respiratory energy is not sufficient to account for the whole of the facts but that some additional factor is operative. What this factor may be remains speculative, but analogy suggests that it may be due to an increase of rate of protein hydrolysis during senescence. Blackman (1928) regarded the senescent "hump" which occurs in the respiration of storage organs during starvation as brought about by "increase in hydrolysis facility" which he pictured as more enzymes coming in contact with their substrates. Godwin and Bishop (1927) showed in starving cherry-laurel leaves that the rate of HCN production from cyanogenetic glucosides greatly increased during yellowing. A similar increase in enzyme availabilty might be invoked to explain the results described here: its effect would be that a higher level of respiration rate would be required to maintain a given amount of protein during the later stages of the life cycle than during the earlier stages.

It will be apparent from the data already presented that amino acids may be regarded not only as substrates for respiration but also from the viewpoint that the amount of amino-acid is determined by the respiration rate. Such a possibility is discussed in the following section.

Suggested Schema of Metabolic Sequences.

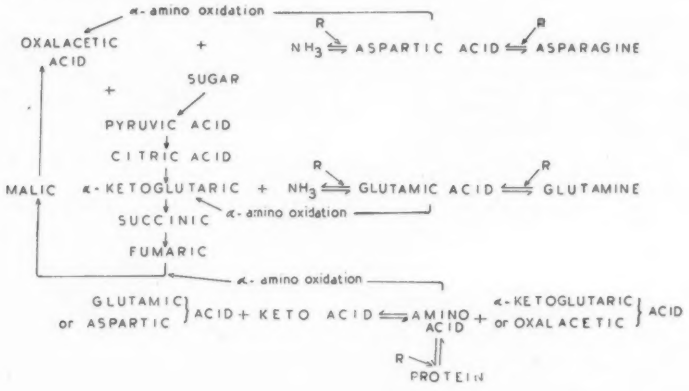
In formulation of suggested metabolic sequences in plant organisms thermodynamic considerations cannot be overlooked. Borsook and Huffmann (1938) have recently drawn attention to the fact that the formation of the peptide linkage of proteins from amino-acids is a reaction in which the free energy increases and that consequently energy for the process must be derived from some linked reaction. Similarly the formation of most amino acids by amination of keto acids are reactions which proceed with free energy increase and therefore are linked with other energy yielding reactions. Amide formation from glutamic and aspartic acid proceeds with a decrease in free energy, however.

These considerations receive point from recent work on animal tissues. Euler (1938) and Adler (1938) have demonstrated an enzyme, glutaminic dehydrogenase, which hydrolyzes glutamic acid to NH_3 and α-ketoglutaric acid. Normally the reaction proceeds until equilibrium is towards the side of complete hydrolysis, but if oxidation of other substances is proceeding simultaneously in the medium then the point of equilibrium is shifted and considerable amounts of glutamic acid are produced from NH_3 and α-ketoglutaric acid.

Coupled with this reaction is the important observation of Braunstein and Kritzmann (1937) that glutamic and aspartic acids in the presence of other keto-acids transfer their amino-group to the keto acids reforming α-ketoglutaric and oxalacetic acid respectively. This reaction has become known as "Transamination" and represents the most likely method of amino-acid formation in plants.

The position of α-ketoglutaric and oxalacetic acids in this scheme, coupled with our findings that citric and malic acids are related to both sucrose and amino-acid contents, and that all four variables are correlated with aerobic and anaerobic respiration focusses attention upon the Krebs cycle. According to Krebs pyruvic acid derived from glycolysis is oxidized in stages, through the agency of the respiratory ferment-cytochrome system, resulting in the formation of a cycle of organic acids which act as "respiratory mediators". The suggested cycle is illustrated in the schema set out below.

The series of reactions outlined above whilst clarifying relations between amino-acids, sugar and organic acids is inadequate to explain fully the respiration rates described in this experiment. In particular it does not account for the observed increase in *OR* with increased amino content due to increased NH_3 content since the same amount of keto-acid would re-enter the cycle after amino-acid formation as left it. It is probable therefore that a direct oxidative deamination of amino-acids takes place yielding ammonia and keto acids some of which enter the organic acid cycle. We have also included in the schema the probable effect of respiratory energy in determining the relations between amino-acids and protein and between amides and amino-acids. The suggested schema is as follows:



The schema accounts for the correlations observed in this experiment between *OR* and *S*, *A* and citric and malic acids. It accounts also for the relations between *NR* and the above variables. In nitrogen the amino-oxidase and cytochrome systems are inoperative and the observed effect of increased amino-acid content on *NR* is probably caused by a shift in the transamination system towards keto acids as a result of protein hydrolysis which takes place as protein content adjusts itself to the new lowered rate of respiration. Keto acids then produce CO_2 by decarboxylation.

SUMMARY.

At intervals through the life cycle of Sudan Grass, respiration rate of leaves was measured in both air and nitrogen by a conductometric method. On comparable leaf material analyses were made of hexoses, sucrose, glutamine, asparagine, amino-acids, total protein, chloroplast protein, citric, malic and oxalic acids.

Both aerobic and anaerobic respiration rates show high linear correlations with cytoplasmic protein-N, but the relation with total protein-N is more complex. It is suggested that cytoplasmic protein-N is a measure of the number of respiratory centres.

Respiration rate on a cytoplasmic basis both in air and in nitrogen shows correlation with amino-acid N, sucrose, malic and citric acid contents. Malic and citric acid contents are also correlated with sucrose and amino-acid contents. In air there is greater utilization of amino-acids than in nitrogen.

It is shown that the observations are accounted for by a schema involving oxidation through the Krebs cycle and transamination; but the data also suggest an oxidative deamination of amino-acids in air, and also that the steady state relation between proteins and amino-acids and between amides and amino-acids may be determined by the respiratory tempo.

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REFERENCES.

Adler, E. *et al.* (1938): C.R. trav. lab. Carls., 22, p. 15.
Amos, G. L. and Wood, J. G. (1939): Austral. J. exp. Biol., 17, p. 285.
Audus, L. J. (1935): New Phytol., 34, p. 386.
Barker, J. (1936): Proc. Roy. Soc. Lond. B., 119, p. 453.
Blackman, F. F. (1928): *Ibid.*, 103, p. 491.
Blackman, F. F. and Parija, P. (1928): *Ibid.*, 103, p. 412.
Borsook, H. and Huffmann, H. M. (1938): Ch. XV in Schmidt's "Chemistry of Amino-Acids and Proteins". Baltimore.
Braunstein, A. E. and Kritzmann, M. G. (1937): Enzymologia, 2, pp. 129, 138.
Euler, H., *et al.* (1938): Z. physiol. Chem., 254, p. 61.
Godwin, H. and Bishop, L. R. (1927): New Phytol., 26, p. 294.
Gregory, F. G. and Baptiste, E. C. D. (1936): Ann. Bot., 50, p. 579.
Gregory, F. G. and Sen, P. K. (1937): Ann. Bot. N.S., 1, p. 521.
Hanson, E. A. (1941): Austral. J. exp. Biol., 19, p. 157.
Hanson, E. A., Barrien, B. S. and Wood, J. G. (1941): *Ibid.*, 19, p. 231.
Petrie, A. H. K. and Williams, R. F. (1938): *Ibid.*, 16, p. 347.
Pucher, G. W., Vickery, H. B. and Wakeman, A. J. (1934): Ind. and Chem. Eng. Anal. Ed., 6, p. 140.
Richards, F. J. (1938): Ann. Bot. N.S., 2, p. 491.
Turner, J. S. (1940): Austral. J. exp. Biol., 18, p. 273.
Vickery, H. B. and Pucher, G. W. (1933): Conn. Agr. Expt. Sta. Bull. No. 352.
Walkley, J. (1940): New Phytol., 39, p. 362.
Wood, J. G. and Petrie, A. H. K. (1938): Ann. Bot. N.S., 2, p. 729.

OBSERVATIONS ON STAPHYLOCOCCAL BETA TOXIN AND ITS PRODUCTION BY STRAINS ISOLATED FROM STAPHYLOCOCCAL LESIONS IN MAN

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Staphylococci may produce at least two exotoxins, α toxin and β toxin. α toxin is lethal for rabbits and mice, is formed by all or nearly all pathogenic strains of human origin, and is regarded as the agent chiefly responsible for the pathological disturbances in staphylococcal infections.

Morgan and Graydon (1936) have shown that this α toxin may consist of two separate components, α_1 and α_2 toxins, and that these are distinct from the β toxin of Glenny and Stevens (1935) which, though noted in their series, was relatively uncommon. They suggested, moreover, that conditions of cultivation influence the production of the α_2 component irrespective of the strain.

β toxin, which causes the "hot-cold" lysis of sheep red cells, is practically non-lethal for rabbits and mice, and is said to occur only rarely in strains of human origin. No strain producing β toxin only has yet been reported as responsible for disease in the human being. Cowan (1938) found that of 82 strains of human origin, only 3 produced β toxin. Christie and Keogh (1940) found 18 of 141 human haemolytic strains produced β as well as α toxin. β toxin occurs more frequently in animal strains (Minett, 1936; Cowan, 1938) and may occur there without the α toxin. It is regarded as of little significance in human infection.

Bryce and Rountree (1936) showed that sheep blood agar was suitable for determining the haemolytic nature of staphylococci. α strains produce on this medium colonies surrounded by zones of clearing. β strains produce colonies surrounded by zones of darkening while $\alpha\beta$ strains produce colonies surrounded by a zone of clearing within a zone of darkening. Rountree (1936) showed that β variants could be produced from some α strains by suitable methods of cultivation.

During an examination of a number of strains in this laboratory, using titration of toxins against rabbit and sheep red cells as well as appearance of colonies on sheep blood agar to identify the toxins, the findings of Bryce and Rountree and of Rountree were confirmed, whilst some additional information regarding β toxin was obtained.

EXPERIMENTS AND RESULTS.

A. *Haemolysis on Sheep Blood Agar Plates.*

Strains of staphylococci were spot-inoculated on sheep blood agar and incubated at 37° C. for 24 hours. The α strains showed zones of complete or nearly complete clearing around their colonies but it was noticed that there were three varieties of these zones:

1. A zone of clearing with a hazy boundary. (See Figure 1 (B).)
2. A zone of clearing with a fairly sharp edge, the medium in this zone appearing glassy-clear as compared with the faintly opaque zone obtained with the first type.
3. An inner zone of clearing surrounded by a zone of partial lysis, even in degree over its whole area, and having quite sharply defined inner and outer margins. (See Fig. 1 (A).)

Cooling for some hours on the bench or at 4° C. followed by a further 24 hours' incubation caused no change in the first variety of zone beyond some extension. The second variety usually developed an outer zone of partial lysis while the third variety showed extension of both the clear and the partly clear zone with in some cases, formation of a third zone a little darker than the

second, but still showing some lysis. Further cooling and incubation accentuated the difference between the first variety and the other two.

The possibility was examined that β toxin, in quantity insufficient to pass beyond the α zone and produce a typical zone darker than the rest of the medium, was responsible for the appearance in the second and third varieties. Considerable evidence was found in support of this view.

Anaerobic growth on sheep blood agar inhibits the appearance of the α zones but still allows the β zones to appear. A pure β strain, a strain of the first variety and a strain of the third variety were grown for three days anaerobically on sheep blood agar. Zones of darkening showed around the colonies of the pure β strain and of the strain of the third variety. No lysis appeared around the colonies of the first variety. That the two zones of darkening were caused by β toxin was confirmed by incubating the colonies for 24 hours further after inoculating a non-haemolytic, lipolytic strain (S36) on the dark zones. In both cases, zones of clearing appeared around the colonies of strain S36 within the zones of darkening (Christie and Graydon, 1941).

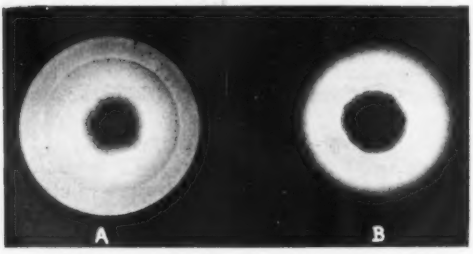

Fig. 1 (A). A colony of staphylococci on sheep blood agar after 24 hours at 37° C., showing an inner zone of clearing and an outer zone of partial lysis, both zones having entire borders (really α(β) haemolysis).

Fig. 1 (B). A colony of staphylococci on same plate as (A) showing the surrounding zone of clearing with a hazy boundary.

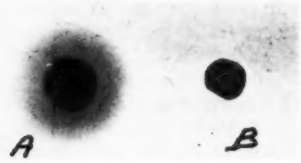

Fig. 2 (A). A colony of staphylococci (S62, an αβ strain) on human serum agar surrounded by zone of opacity.

Fig. 2 (B). A colony of staphylococci (S210, a pure α strain) on same plate with no change in surrounding medium.

A two-months-old agar slope culture of this strain of the third variety was plated out on sheep blood agar to give single colonies. Several of these were definite αβ variants while some of the others were of the second variety, thus suggesting that strains of the second variety were essentially the same as those of the third variety but produced less β toxin. That the appearance in strains of the second variety is due to α and β toxin is suggested by the findings of Christie and Graydon (1941) who showed that β toxin may augment the action of α toxin on sheep red cells.

Support for the view that the outer partly-lysed zone shown by the third variety of strains was due to β toxin was obtained by plate experiments with cell-free toxins. A drop of pure β toxin caused a zone of darkening on a sheep blood agar plate while a drop of α toxin caused a zone of clearing with a hazy edge. Mixtures of α and β toxin were prepared, ranging from 5 parts of α toxin and 95 parts of β toxin at the one end to 95 parts of α toxin and 5 parts of β toxin at the other. Drops of these mixtures were allowed to fall on a blood agar plate and the plate left at 37° C. for an hour. Where the β toxin was in excess a dark zone formed. As the α toxin increased, a zone of partial clearing appeared in the centre of the dark zone until a point was reached where the outer zone instead of being darker than the rest of the medium, appeared lighter. This was identical with the appearance of the third variety described above. With still lower proportions of β toxin appearances approximating to those of the second variety were observed.

Seventy-two old strains, originally recorded as showing α haemolysis from the appearance of their colonies on sheep blood agar were spot-inoculated on sheep blood agar, alternately incubated and cooled daily for a few hours and examined at intervals. Seven gave the definite appearance of αβ strains, the outer zone being darker than the rest of the medium, 14 gave the appearance of the first variety and the other 51 were of the second and third varieties. The strains were plated out on sheep blood agar, incubated for 24 hours and examined. 22 of the strains showed definite α β variants.

Agar slope cultures of each strain were prepared from single colonies which showed no definite signs of darkening indicative of β toxin. Efforts were made to prepare αβ variants from these by growing in broth under increased CO_2 tension, in peptone water and anaerobically in broth for several days. We were able to obtain either αβ variants or variants of the third variety from all

except 15 of the strains. Many of the strains gave variants showing β and no α toxin. Non-haemolytic and visibly rough colonies also appeared.

These results suggest that β toxin is more commonly produced by staphylococci of human origin than was formerly thought, and that most strains generally regarded as solely α toxin producers are either actual or potential producers of β toxin as well. It will be convenient to refer to the strains of the second and third varieties as α(β) strains.

Strain "Mills" and strain "Denis", two of the strains used by Morgan and Graydon (1936) in detecting the two components of α toxin, were examined. Strain "Mills" (α1) was of the first variety, i.e. a pure α strain. Strain "Denis" (α1 α2) was an α(β) strain. It seemed not improbable that the β toxin in strain "Denis" might have had an influence on the rabbit red cell lysis by α toxin similar to the α and β lysis of sheep red cells found by Christie and Graydon (1941) and that α2 toxin was actually β toxin. This view was supported by Roy's (1937) statement that β toxin affects the neutralization power of α antitoxin for α toxin. If this were so, however, the addition of β toxin to α toxin ought to increase the degree to which the latter will lyse rabbit cells. This was tested by adding varying quantities of β toxin to a fixed quantity of α toxin and titrating the mixtures so obtained against rabbit cells. The results indicated that β toxin had no effect on the lytic power of α toxin for rabbit red cells. Thus there was no evidence that α2 and β toxins were the same.

Since all the strains used in these tests had been maintained on artificial media for a considerable time, it was possible that the instability of the strains and tendency to form haemolytic variants were peculiarities which would not be shown by freshly-isolated strains. To test this, strains were obtained from boils and examined without delay. Of the 43 strains examined, 39 were of the α(β) variety and 8 of these showed definite αβ variants on the primary isolation plate. Only 4 were pure α strains (first variety).

B. *Haemolysis on Human Blood Agar Plates.*

Strain "Mills" which produces α toxin only and strain S32a (received from Dr. L. Bryce) which produces β toxin only were spot-inoculated on 2·5 p.c. human blood agar and incubated at 37° C. After 4 days no haemolysis showed around either colony. On further incubation a small zone of partial lysis formed around strain "Mills" with an outer zone of slight browning, whilst a narrow zone of very weak haemolysis formed around strain S32a. On the seventh day a zone of reddish darkening of considerable extent formed around the narrow inner zone; this resembled the β zone which forms on sheep blood agar within 24 hours but was fainter and was not affected by subsequent refrigeration. It was evident that the haemolysis seen and reported on human blood agar had a different significance from that caused by α or β toxin on sheep blood agar. Strains whose haemolytic nature was known from the appearance of their colonies on sheep blood agar were incubated on human blood agar plates. The only strains which showed zones of complete haemolysis within 24 hours were the definite αβ strains. Those showing zones of partial clearing within 24 hours were of the α(β) type, many of these zones becoming clear on longer incubation. One strain which produced much β toxin and little α toxin showed only a small zone of slight haemolysis on the third day with little change on further incubation until the fifth day when the wide zone of slight darkening, apparently indicative of β toxin, appeared.

C. *The Action of β Toxin on Human Serum.*

Strains of staphylococci were spot-inoculated on human serum agar plates and incubated at 37° C. Zones of opacity appeared in the otherwise clear medium around some of the colonies. Only strains producing much β toxin caused these zones to form. (See Fig. 2.) Thirty-nine animal strains were similarly inoculated. The 35 αβ strains caused definite zones whilst the 4 α strains did not.

Strain "Mills" caused no zone but strain S32a, the pure β strain, gave a marked zone.

The assumption that the effect was caused by β toxin was confirmed by placing a drop of cell-free β toxin on the medium and incubating. Within 4 hours a zone of opacity marked the spot where the β toxin was placed. A drop of β toxin neutralized by β anti-toxin caused no opacity; normal serum did not inhibit the phenomenon. A drop of α toxin had no effect on the medium.

A cell-free piece of agar cut from a turbid zone around a colony and placed on a second plate produced a second zone of opacity on the fresh medium. A piece of this turbid agar placed on a third plate caused a third zone of opacity.

The opacity could be produced in liquid serum in test-tubes by adding β toxin to it. On diluting the toxin, however, the power to cause the opacity in liquid serum was readily lost. The opacity did not appear on plates made from sheep, rabbit, guinea-pig or horse serum. In this sense as well as in the fact that various human samples differed in their sensitivity to the toxin, the reaction resembled the action of Cl. welchii toxin on serum (Nagler, 1939). To investigate the possibility as to whether the action of the two toxins was on the same constituent of the serum, three samples of human serum were tested against falling dilutions of β toxin and of Cl. welchii toxin. Degree of sensitivity to β toxin did not parallel the degree of sensitivity to Cl. welchii toxin.

Serum agar plates made with 20 p.c. serum were found to be most suitable for the test. Seitz-filtration, inactivation and heating for one hour at 65° C. did not render the serum unsuitable for the test.

A piece of the opaque agar examined microscopically showed that the opacity was due to the presence of minute amorphous particles such as are formed when a weak albumin solution is heated. To find out which constituent of the serum was responsible for the reaction, the albumin and globulin were separated out by the usual fractionation methods using ammonium sulphate. The albumin and globulin were re-dissolved to give twice their former concentration. Albumin agar and globulin agar plates were prepared as well as albumin-globulin agar plates in which the albumin and globulin were present in the concentrations in which they were present in serum agar. The opacity appeared much denser on the albumin than on the globulin plates.

Agar plates to which egg albumin had been added did not show the opacity.

Small zones of opacity formed around many of the colonies other than those referred to above. These differed from the zones formed by β toxin in that they had a faint bluish tinge and not a faint brownish one; they were very narrow and did not tend to increase after 24 hours; they had a sharp edge, not a hazy one, and appeared to some extent on serum other than human serum.

A plate of serum agar containing a zone of opacity caused by a drop of cell-free β toxin was cooled at 4° C. for an hour and re-incubated. A second zone appeared outside the first with a fine zone of clear medium between the two zones. This resembled the action of β toxin on sheep blood agar; a circular line of apparently unaltered blood marks the division between the two zones of β haemolysis formed under similar circumstances.

A third appearance given only by strains of the third variety described above showed only after 3 or 4 days' incubation. Narrow concentric zones of marked opacity formed around the colonies of these strains on human serum agar and albumin agar.

DISCUSSION.

The observations recorded above showing the frequency with which actual or potential β toxin formation can be demonstrated in strains of staphylococci isolated from lesions in human beings, are of some interest. They tend to confirm Rountree's (1936) work and extend it by showing that the bulk of strains isolated from boils form β as well as α toxin. Bryce and Rountree (1936) found that almost all of 65 random Wassermann samples of serum from human adults had a definite β anti-toxin content. It is possible that previous infection with β toxin-forming staphylococci was responsible for this anti-toxin.

The specific action of β toxin on human serum suggests the possibility of a reliable test for $\alpha\beta$ toxin-producing strains in which the β toxin is insufficient to give the typical appearance round the colonies on sheep's blood agar plates.

Further, some speculations as to the possible rôle played by β toxin in human staphylococcal infections appear to be justified. Gengou (1933), as a result of very convincing experiments, concluded that the clotting of plasma and the subsequent digestion of the fibrin (fibrinolysis) were simply stages in the one process and caused by a single substance or enzyme secreted in the staphylococci. This is the substance known as coagulase. This process of clotting followed by digestion of the fibrin was found by Gengou to be influenced by the concentration of globulin and more particularly of the albumin in the plasma. With high concentration of these proteins the process stopped short at the stage of coagulation. Lowering their concentration hastened the process, so that in some cases the clotting stage might be missed altogether.

It has been suggested that localization of staphylococcal infection is caused by the coagulase of the organism. Menkin and Walston (1934–35), however, have shown that by precipitation with glacial acetic acid, the coagulase may be separated out from a staphylococcal filtrate, free from the principle which causes lymphatic blockage.

As a big proportion of strains of staphylococci immediately after isolation from boils (39 out of 43) were found to produce at least some β toxin in addition to α, it is reasonable to suspect that the β toxin may play some rôle in the evolution of the lesion. It is suggested that the β toxin formed by the infecting cocci may assist in inhibiting the spread of the lesion by denaturation of the tissue proteins

and/or serum proteins, especially the albumin. At the same time the staphylococci are secreting an enzyme, coagulase which, by clotting fibrinogen, may help in the early stage to wall off the lesion. As the concentration of the albumin and globulin in solution is lowered by the precipitating action of the β toxin, the fibrin is digested and liquefaction ensues. Further work would be necessary to substantiate or disprove this hypothesis.

The mechanism by which β toxin augments the lytic action of α toxin on human erythrocytes is not clear, and an explanation of the phenomenon will not be attempted at the present juncture.

SUMMARY.

In an examination of 72 old strains of α toxin-producing staphylococci, it has been shown that 58 were either actual or potential producers of β toxin. Of 43 strains examined immediately after isolation from boils, 39 were found to produce β toxin.

A plate method using sheep blood agar for detecting the difference between such strains and strains which produce only α toxin, is described.

β toxin has been shown to cause an opacity in human serum which is probably due to denaturation of the serum proteins.

β toxin has been shown to cause haemolysis of human red cells in human blood agar in the presence of sufficient α toxin within 24–48 hours, although neither toxin will do so alone.

One possible rôle of β toxin in the evolution of lesions such as boils is suggested.

Acknowledgment. We are indebted to Mr. F. J. Dempster for preparing the photographs.

REFERENCES.

Bryce, L. M. and Rountree, P. M. (1936): J. Path. Bact., 43, p. 173.
Christie, R. and Graydon, J. J. (1941): Austral. J. exp. Biol., 19, p. 9.
Christie, R. and Keogh, E. V. (1940): J. Path. Bact., 51, p. 189.
Cowan, S. T. (1938): *Ibid.*, 46, p. 31.
Gengou, O. (1933): Annales de l'Institut Pasteur, 51, p. 14.
Glenny, A. T. and Stevens, M. F. (1935): J. Path. Bact., 40, p. 201.
Menkin, V. and Walston, H. D. (1934–5): Proc. Soc. exp. Biol., 32, p. 1259.
Minett, F. C. (1936): J. Path. Bact., 42, p. 247.
Morgan, F. G. and Graydon, J. J. (1936): *Ibid.*, 43, p. 385.
Nagler, F. P. O. (1939): Brit. J. exp. Path., 20, p. 473.
Rountree, P. M. (1936): Austral. J. exp. Biol., 14, p. 93.
Roy, T. E. (1937): J. Immunol., 33, p. 437.

A TEST OF STAPHYLOCOCCAL FIBRINOLYSIS

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The technique of Tillett and Garner (1933) for demonstrating streptococcal fibrinolysis appears to be unsuited to staphylococci on account of the length of time taken by some strains to liquefy the clot, the uncertainty of the results when the test is repeated and the occurrence of retraction or of liquefaction of the sterile uninoculated control clot during prolonged incubation. The reports of Madison (1935–6), Fisher (1936), Neter (1937) and Saski and Fejgin (1937) suggest that fibrinolysis goes with coagulase production and is characteristic of pathogenic staphylococci of human origin.

A test is here described in which lysis by staphylococci of heat-precipitated fibrinogen incorporated in nutrient agar may be observed. Among staphylococci of human origin such lysis was caused only by pathogenic strains.

Materials and Methods.

Nutrient agar was melted and cooled to about 50° C., 12 p.c. of sterile oxalated plasma was added, the mixture was heated at 56° C. for 10–15 minutes in order to precipitate the fibrinogen and plates were poured. This medium was turbid.

Spot-inoculation was done by touching the surface of the medium once with a platinum loop charged with staphylococci. By this means six strains were grown readily on a 9 cm. plate. Incubation was at 37° C.

One hundred and forty-one strains of human origin and 57 strains of animal origin were examined. Coagulase tests were carried out as previously described (Christie and Keogh, 1940). Fibrinolysis was tested for by the technique of Tillett and Garner. α and β toxins were recognized by cultivation on sheep blood-agar plates (Bryce and Rountree, 1936) and, in many cases, by the action on sheep red cells of sterile filtrates of four days' "sloppy agar" cultures which had been incubated under increased CO_2 tension. Dermonecrosis was demonstrated by injecting similar culture filtrates into the skin of rabbits. Leucocidin was demonstrated for us by Dr. E. A. North using Valentine's (1936, 1939) technique.

TABLE 1.

Staphylococci of human origin.

Relationship between clearing of fibrinogen-agar, coagulase and kind of toxin.

	Coagulase.		
Fibrinogen-agar.	Positive.	Negative.	Total.
Clearing	92 (α, 90; neg., 2)	0	92
No clearing	7 (α, 1; $\alpha\beta$, 5; neg., 1)*	42 (all neg.)	49
	99	42	141

α, $\alpha\beta$, β or negative, in parenthesis, refer to the number of strains which produced the respective toxin or which showed negligible or no haemolysis.

* The α strain (S39, mentioned in the text) and the "negative" strain both cleared rabbit fibrinogen-agar.

Results.

After incubation overnight on fibrinogen-agar plates the colonies of some strains were surrounded by zones of clearing, which became wider after further incubation, and in which the precipitated fibrinogen was digested or dissolved, whereas the medium surrounding colonies of

other strains retained its original turbidity (Fig. 1, A and B). A few strains which failed to cause clearing overnight did so after further incubation up to 48 hours.

Plates made with rabbit plasma were more sensitive than those containing human, sheep or horse plasma. The results here described were obtained with sheep plasma.

Many strains which caused clearing of fibrinogen-agar failed to liquefy the fibrin clot which resulted when calcium chloride was added to oxalated plasma or to liquefy the fibrin clot which they themselves had produced by means of coagulase, but all strains which liquefied fibrin clot caused clearing of fibrinogen-agar. The results of fibrinogen-agar and coagulase tests are shown in Tables 1 and 2.

TABLE 2.

Staphylococci of animal origin.

Relationship between clearing of fibrinogen-agar, coagulase and kind of toxin.

	Coagulase.		
Fibrinogen-agar.	Positive.	Negative.	Total.
Clearing	4 (all α)	1 (negative)	5
No clearing	39 (all αβ or β)	13 (all neg.)	52
	43	14	57

α, αβ, β or negative, in parenthesis, refer to the number of strains which produced the respective toxin or which showed negligible or no haemolysis.

The following considerations suggest that clearing of fibrinogen-agar is indicative of fibrinolysis:

(i) The same results were observed when a solution of fibrinogen in physiological saline was substituted for plasma.

(ii) As shown in Table 3 there is some agreement between the results of the two tests, using human plasma, as applied to β haemolytic streptococci of human origin. We are indebted to Mr. R. T. Simmons for applying the test of Tillett and Garner to the streptococci. In groups A and B there was complete agreement; in groups C and G, however, some strains which liquefied fibrin clot failed to cause clearing of fibrinogen-agar. Cultivation on fibrinogen-agar is not recommended as a test of fibrinolysis by streptococci for which the technique of Tillett and Garner is more satisfactory. The zones of clearing surrounding colonies of streptococci were not as obvious as those surrounding colonies of staphylococci.

(iii) Other workers (mentioned above) have found indications that fibrinolysin is characteristic of pathogenic staphylococci of human origin. Our results with strains of human origin (Table 1) show a similar but not invariable connection between pathogenicity (as judged by coagulase production) and clearing of fibrinogen-agar. The few exceptions in this group and the many exceptions among strains of animal origin (Table 2), which will be discussed below, are associated with the presence of β toxin.

TABLE 3.

Beta haemolytic streptococci of human origin.

Relationship between fibrinolysis (Tillett and Garner) and clearing of fibrinogen-agar.

Group.	Fibrinolysis and clearing.	No fibrinolysis and no clearing.	Fibrinolysis but no clearing.	Total.
A	39	2	0	41
B	0	21	0	21
C	6	3	3	12
G	6	0	12	18
			Total	92

Relation to α and β toxins. Tables 1 and 2 show that of the 97 strains which caused clearing of fibrinogen-agar, 94 formed α toxin, 3 formed little or no toxin. Of the 101 strains which did not cause clearing of fibrinogen-agar, 44 formed β toxin, 56 were non-haemolytic, one formed α toxin. The width of the clearing around colonies on fibrinogen-agar was, however, not invariably proportionate to the width of the zones of α haemolysis around colonies on sheep blood-agar. Moreover,

the one coagulase positive strain (S39) which formed α toxin but which did not cause clearing of fibrinogen-agar (Table 1) was a strong producer of α toxin[1]; α toxin, therefore, was apparently not identical with the factor causing clearing of fibrinogen-agar, although clearing of fibrinogen-agar was generally associated with the presence of α toxin.

Whether α toxin were present or not, no strain which formed β toxin in appreciable quantity caused clearing of fibrinogen-agar, suggesting that β toxin prevented clearing of fibrinogen-agar in the same way as it prevents lysis of sheep red cells by α toxin (Christie and Graydon, 1941). The following experiment, however, seemed to disprove this suggestion: A strain producing β toxin only was cultivated for 48 hours on the surface of fibrinogen-agar. No clearing occurred. A strain producing α toxin was then grown close to a colony of that strain. The clearing caused overnight by this latter strain (producing α toxin) was as great as that caused by colonies of this strain grown on another plate of fibrinogen-agar which was free from β toxin.

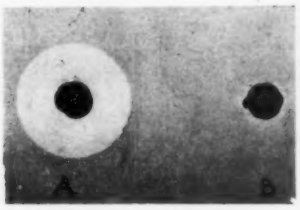

Fig. 1. Photograph of colonies, actual size, of staphylococci after 48 hours' cultivation on the surface of fibrinogen-agar: A, clearing; B, no clearing.

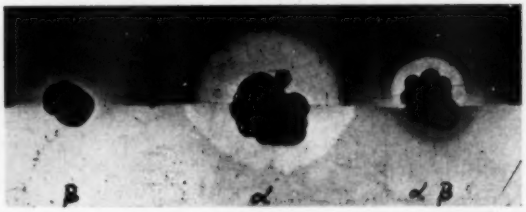

Fig. 2. Photograph of colonies, actual size, of coagulase-positive strains of staphylococci after 40 hours' cultivation on the junction between 5 p.c. sheep blood-agar in one half of a Petrie dish and fibrinogen-agar in the other half of the dish.

Colonies producing α, αβ or β toxin have caused the usual appearance in sheep blood-agar but only the colony which is free from demonstrable β toxin has caused clearing of the fibrinogen-agar.

Relation to coagulase. Only one of the 97 strains which caused clearing of fibrinogen-agar was coagulase-negative. This strain (S159) was isolated from a cow's udder; it produced no α or β toxin, it was not agglutinated by any available serum (Christie and Keogh, 1940; Christie, 1940) and it was strongly caseinolytic. The zone of clearing around this singular strain differed from that around all other strains in that it had a diffuse, hazy outer margin in contrast to the sharp margin of other strains (Fig. 1) suggesting that the factor responsible for clearing by strain S159 was different from that responsible for clearing by the remaining 96 strains.

Forty-six of the 101 strains which failed to cause clearing of fibrinogen-agar were coagulase-positive but 44 of these produced β toxin; one of the remaining two strains produced neither α nor β toxin, one produced α toxin.

DISCUSSION.

These observations appear to indicate that the factor "F", responsible for clearing of fibrinogen-agar is associated with coagulase and with α toxin and that it is inhibited by, or its effect is masked by, β toxin, a relationship which may be stated thus, $\alpha F \rightarrow \alpha(\beta)F \rightarrow \alpha\beta \rightarrow \beta$ and which is illustrated by Fig. 2.

There are, however, among the 198 strains referred to in Tables 1 and 2, four exceptions to this hypothetical relationship between fibrinolysin, coagulase and α and β toxins: of the 97 strains which caused clearing of fibrinogen-agar all but three produced both coagulase and α toxin without demonstrable β toxin; of the 101 strains which failed to cause clearing of fibrinogen-agar one produced both coagulase and α toxin without demonstrable β toxin.

Clearing of fibrinogen-agar was apparently unrelated to leucocidin, dermonecrotoxin or to the enzyme which causes liquefaction of gelatine.

If strains of human origin only are considered, clearing of fibrinogen-agar is

[1] But see footnote to Table 1.

an indication of pathogenicity. Exceptions to this relationship are found with strains producing much β toxin, but such strains are rare in human lesions. The experience of Madison (1935-6), who found that many strains of human origin, but no strains of animal origin, were fibrinolytic, supports this contention, since most pathogenic animal strains produce much β toxin.

Cultivation on fibrinogen-agar of recently isolated staphylococci of human origin is suggested as a possible test of pathogenicity. It affords, in most instances, indirect evidence of the presence of α toxin, coagulase and fibrinolysin.

SUMMARY.

Nutrient agar, rendered turbid by the presence of heat-precipitated fibrinogen, was used as a means of detecting fibrinolysis by staphylococci. Zones of clearing surrounding colonies on the surface of this medium are believed to indicate the presence of fibrinolysin.

Ninety-two out of 99 coagulase-positive strains of human origin, but none of 42 coagulase-negative strains caused clearing of fibrinogen-agar. Of strains of animal origin, 4 out of 43 coagulase-positive strains and one out of 14 coagulase-negative strains caused clearing.

In both groups most of the coagulase-positive strains which failed to produce clearing were strong producers of β toxin; the discrepancy between the results with human and animal strains is related to the comparative rarity of β toxin in the former and to its frequency in the latter.

With human strains, clearing of fibrinogen-agar generally runs parallel with the presence of coagulase and with pathogenicity.

Acknowledgment. We are indebted to Mr. F. J. Dempster for preparing the photographs.

REFERENCES.

Bryce, L. M. and Rountree, P. (1936): J. Path. Bact., 43, p. 173.
Christie, R. and Keogh, E. V. (1940): *Ibid.*, 51, p. 189.
Christie, R. (1940): Austral. J. exp. Biol., 18, p. 397.
Christie, R. and Graydon, J. J. (1941): *Ibid.*, 19, p. 9.
Fisher, A. M. (1936): Bull. Johns Hopkins Hosp., 59, p. 415.
Madison, R. R. (1935–6): Proc. Soc. exp. Biol., 33, p. 209.
Neter, E. (1937): J. Bact., 34, p. 243.
Saski, S. and Fejgin (1937): C.R. Soc. Biol., 126, p. 139.
Tillett, W. S. and Garner, R. L. (1933): J. exp. Med., 58, p. 485.
Valentine, F. C. O. (1936): Lancet, 230, (1), p. 526.
Valentine, F. C. O. and Butler, E. C. B. (1939): *Ibid.*, 236, (1), p. 973.